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Cell Wall Degrading Enzymes of the Plant Pathogenic Fungus *Stagonospora nodorum*

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Cell Wall Degrading Enzymes of the Plant Pathogenic Fungus *Stagonospora nodorum*

Submitted by Amanda Jane Carlile
for the degree of PhD
of the University of Bath
1999

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Table of Contents

Abstract	1
Abbreviations	2
Chapter One: General Introduction	4
1.0 'Septoria' diseases	4
1.0.1 Disease symptoms	5
1.0.2 Disease cycle	5
1.0.3 Host specificity	8
1.0.4 Geographic distribution and losses	9
1.0.5 Identification and diagnosis	10
1.0.6 Host colonisation	10
1.1.6.1 Adhesion and germination	11
1.1.6.2 Host penetration	13
1.0.6.3 Host colonisation and resistance	15
1.0.7 Control	17
1.1 Rational Design of Fungicides	18
1.1.1 Fungicide research and development	18
1.1.2 Biorational fungicide design	20
1.2 Pathogenicity factors - Fungicide Targets?	26
1.2.1 Detoxification of pre-formed antifungal molecules	29
1.2.2 Detoxification of phytoalexins	29
1.2.3 Toxin production	29
1.2.3.1 Non specific toxins	30
1.2.3.2 Host selective toxins	30
1.2.4 Cell wall degrading enzymes	30
1.3 The Plant Cell Wall	31
1.3.1 Function of the wall	32
1.3.2 Structure, composition and enzymic degradation of wall polymers	32
1.3.2.1 The microfibrillar domain	33
1.3.2.2. The matrix domain	34
1.3.2.2.1 Pectic polysaccharides	37
1.3.2.2.2 Xyloglucan	37
1.3.2.2.3 Glucuronoarabinoxylan	38
1.3.2.2.4 β 1,3-, β 1,4-glucans	40
1.3.2.2.5 Proteins	41
1.3.2.2.6 Lignin and other phenolic compounds	44
1.3.3.3 Linkages between different wall polymers	45
1.3.2.3.1 Covalent linkages	45
1.3.2.3.2 Non-covalent linkages	45

1.4	Cell Wall Degrading Enzymes in Plant Pathogenesis	46
1.4.1	Cutinases	50
1.4.2	Pectinases	52
1.4.3	Xylanases	54
1.4.4	Glucanases	56
1.4.5	Cellulases	56
1.4.6	Proteases	57
1.4.7	Problems with disruption of genes encoding cell wall degrading enzymes	59

Chapter Two: Materials and Methods **61**

2.0	Maintenance and Culturing of <i>S.nodorum</i>	61
2.0.1	Fungal isolate	61
2.0.2	Preparation and storage of spore stocks	61
2.0.3	Growth of <i>S.nodorum</i> on agar plates	61
2.0.4	Growth of <i>S.nodorum</i> in liquid culture	62
2.0.5	Assessment of fungal growth	62
2.1	Growth and Inoculation of Plants	63
2.1.1	Plants	63
2.1.2	Production of wheat cell walls	63
2.1.3	Whole plant inoculations	63
2.1.4	Detached leaf inoculations for western blot analysis	64
2.1.5	Extraction of enzymes from plant material	64
2.1.6	Effect of inhibitors on disease symptoms	65
2.1.7	Effect of inhibitors on spore germination <i>in vivo</i>	66
2.1.8	Infiltration of antibody solution into wheat leaves	66
2.1.9	Culture of tritordeum suspension cells	67
2.2	Enzyme assays	68
2.2.1	Nelson-Somoygi reducing sugar assay	68
2.2.2	Remazol Brilliant Blue xylanase assay	69
2.2.3	Protease assays	70
	2.2.3.1 Azocasein	70
	2.2.3.2 Trypsin assay	70
	2.2.3.3 Aspartic protease assay	70
2.2.4	Pectin Lyase	71
2.2.5	Glycosidases	71
2.2.6	Carbohydrate assay	71
2.3	Enzyme Characterisation by Isoelectric Focusing	72
2.3.1	Gel isoelectric focusing	72
2.3.2	Protease overlay detection in IEF gels	72
2.3.3	Xylanase overlay detection in IEF gels	73
	2.3.3.1 RBB-dyed xylan overlay	73
	2.3.3.2 Oat spelt xylan overlay	73
2.3.4	Preparative Rotofor™ IEF	73

2.4	Purification of SNP1	74
2.4.1	Ammonium sulphate precipitation	74
2.4.2	Partial purification of an alkaline protease	74
2.4.3	Purification of a trypsin-like protease (SNP1)	75
2.5	Protein Electrophoresis and Western Blotting	76
2.5.1	Protein assay	76
2.5.2	SDS-PAGE	76
2.5.3	Preparation of antibodies	77
2.5.4	Electroblotting	77
2.5.5	Western blot analysis	78
2.6	Molecular Methods	79
2.6.1	Preparation of genomic DNA	79
2.6.2	DNA restriction and agarose gel electrophoresis	79
2.6.3	PCR primers	80
2.6.4	PCR isolation of the <i>try1</i> gene and <i>SNP1</i>	81
2.6.5	Southern blotting and hybridisation	82
2.6.6	Plating cells	83
2.6.7	Heterologous library screen with the <i>try1</i> gene	83
2.6.8	Lambda DNA preparation	85
2.6.9	Subcloning	86
2.6.10	Plasmid DNA preparation	87
2.6.11	Sequencing	87
2.6.12	Isolation of full length <i>SNP1</i>	88

Chapter Three: Cell Wall Degrading Enzymes Produced by *Stagonospora nodorum* **89**

3.0	Introduction	89
3.1	Results	91
3.1.1	Cell wall degrading enzymes produced by <i>S.nodorum</i> <i>in vitro</i>	91
3.1.2	Cell wall degrading enzymes produced by <i>S.nodorum</i> <i>in planta</i>	94
3.1.3	Detection of CWDE in cirrus mucilage	101
3.2	Discussion	
3.2.1	CWDE produced <i>in vitro</i>	103
3.2.2	CWDE produced <i>in vivo</i>	108
3.2.3	CWDE in cirrus mucilage	111

Chapter Four: Proteases of *S.nodorum* 116

4.0	Introduction	116
4.1	Results	122
4.1.1	Regulation of protease activity <i>in vitro</i>	122
4.1.2	Use of inhibitors to classify protease activity	124
4.1.3	Detection of an aspartic protease	124
4.1.4	Isoelectric focusing determination of protease isoforms	129
4.1.4.1	Rotofor™ Preparative Isoelectric focusing	129
4.1.4.2	Gel isoelectric focusing	132
4.1.5	Partial purification and characterisation of an alkaline protease	132
4.1.6	Purification of a trypsin-like protease (SNP1)	136
4.1.7	N-terminal sequence	138
4.1.8	Isolation and characterisation of <i>SNP1</i>	139
4.1.8.1	Heterologous library screen	139
4.1.8.2	PCR amplification of a SNP1 fragment	140
4.1.8.3	Isolation of full length SNP1 genomic sequence	142
4.1.9	Regulation of SNP1	148
4.1.10	Temperature and pH optima	151
4.1.11	Effect of metal ions	151
4.1.12	Inhibitor characteristics	151
4.1.13	Substrate range and kinetics	153
4.1.14	Effect of SNP1 on wheat cell walls	155
4.1.15	Detection of SNP1 <i>in planta</i>	157
4.1.16	Detection of SNP1 <i>in planta</i> and both SNP1 and the aspartic protease in cirrus mucilage	161
4.1.17	Effect of inhibition of protease activity on disease	164
4.1.17.1	Effect of protease inhibitors on infection	164
4.1.17.2	Effect of inhibitors on germination	167
4.1.17.3	Effect of anti-SNP1 antibody on infection	170
4.1.18	Effect of SNP1 on tritordeum suspension cells	173
4.2	Discussion	175
4.2.1	Characterisation of protease activity present in culture filtrate	175
4.2.2	Purification of an alkaline trypsin-like protease	178
4.2.3	Protease regulation	180
4.2.4	Isolation of <i>SNP1</i>	184
4.2.5	Detection of SNP1 <i>in planta</i>	187
4.2.6	Effect of inhibition of protease activity on disease	190
4.2.7	Degradation of cell wall proteins by SNP1	196
4.2.8	Effect of SNP1 on tritordeum cells	199
4.2.9	Other possible roles for proteases in pathogenicity	201

Chapter Five: Xylanases of <i>S.nodorum</i>	204
5.0 Introduction	204
5.1 Results	209
5.1.1 Regulation of xylanase production by carbon sources <i>in vitro</i>	209
5.1.2 Characterisation of the isoforms of xylanase produced by <i>S.nodorum</i> on wheat cell walls	209
5.1.2.1 Rotofor preparative isoelectric focusing	212
5.1.2.2 Gel isoelectric focusing	212
5.1.3 Identification of a high pI xylanase	213
5.1.4 Temperature and pH optimum of Xyl1	217
5.1.5 Confirmation of endo-action of Xyl1	217
5.1.6 N-terminal sequencing of Xyl1	220
5.1.7 Generation of internal protein fragments	221
5.1.8 Production of antibodies to Xyl1	223
5.1.9 Carbon regulation of Xyl1	223
5.1.10 Detection of Xyl1 in infected plant tissue	226
5.1.11 Effect of Xyl1 on wheat cell walls	228
5.1.11.1 Detection of reducing sugar release	228
5.1.11.2 Detection of phenolic-linked polysaccharides	230
5.1.12 Effect of Xyl1, with and without SNP1 on tritordeum suspension cells.	232
5.1.13 The effect of anti-Xyl1 antibody on disease symptoms	234
5.2 Discussion	236
5.2.1 Xylanase regulation	236
5.2.2 Xylanase multiplicity	240
5.2.3 Characteristics of a partially purified high pI xylanase (Xyl1).	245
5.2.4 Detection of Xyl1 <i>in planta</i>	247
5.2.5 Wheat cell wall degradation by Xyl1	249
5.2.6 Effect of Xyl1 on tritordeum suspension cells	252
Chapter Six: General Discussion	256
6.1. Future Work	260
Bibliography	265

Abstract

Cell wall degrading enzymes (CWDE) produced by plant pathogens may be necessary for penetration and colonisation of the host. As pathogenicity factors they would therefore be potential fungicide targets. *Stagonospora nodorum* produced a range of CWDE during growth in liquid culture on wheat cell walls. The same CWDE activities were also detected in the cirrus mucilage surrounding spores produced *in vitro*. Two of the major enzymes *in vitro*, xylanase and protease were also detected at elevated levels in infected plants.

A number of acidic pI (3.8-6.8) xylanase isoforms were detected in cell wall-grown cultures and an alkaline (pI>10), endo-acting xylanase (Xyl1), molecular weight 30kDa, was partially purified by cation exchange chromatography. Xyl1 was induced by xylan, repressed by glucose and xylose and was optimally active at 50°C and at pH 7.0. Xyl1 could degrade the wheat cell wall and was present during later stages of infection.

A trypsin-like protease (SNP1) pI (8.7) and two aspartic proteases (pI 2.6 and 4.5) were detected in cell wall grown cultures. Both protease types were regulated by derepression and induction. SNP1, which possessed the conserved fungal trypsin N-terminus was purified 417-fold to homogeneity by two steps of cation exchange chromatography. SNP1 had a molecular weight of 25kDa and was optimally active at 35°C and at pH 8.5. SNP1 had a trypsin-like substrate specificity, was strongly inhibited by trypsin inhibitors and was stimulated by calcium ions. The *SNP1* gene was isolated from a lambda library and had extensive homology to other tryptins. SNP1 was present throughout infection and released hydroxyproline-containing fragments from the wheat cell wall *in vitro*. Addition of the trypsin inhibitors leupeptin and aprotinin to spores delayed and reduced disease symptoms as did infiltration of wheat leaves with anti-SNP1 antibody.

This evidence suggests that both Xyl1 and SNP1 may play a role in the infection process.

Abbreviations

A	Adenine, deoxyribonucleotide base
Abs	Absorbance
bp	nucleotide base pair
C	cytosine, deoxyribonucleotide base
<i>ca.</i>	<i>circa</i>
CWDE	cell wall degrading enzymes
Da	dalton
dicot	dicotyledoneous plant
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
EDTA	ethylenediaminetetraacetic acid
G	guanine, deoxyribonucleotide base
H	A or C or T
h	hour
IEF	isoelectric focusing
Kat	Katal
kb	kilobase
K _m	Michaelis constant
l	litre
μ	micro
m	milli
M	molar
Mb	megabase
MES	2-(N-morpholino) ethanesulphonic acid
min	minute
monocot	monocotyledoneous plant
N	G or A or C or T
ORF	open reading frame
PCR	polymerase chain reaction

PEG	polyethylene glycol
PG	polygalacturonase
pI	isoelectric point
PL	pectin lyase
PMSF	phenylmethanesulphonyl fluoride
R	A or G
RNA	ribonucleic acid
rpm	revolutions per minute
S	G or C
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
T	thymine, deoxyribonucleotide base
Tris	Tris(hydroxymethyl) aminomethane
V_{\max}	maximum reaction velocity
W	A or T
w/v	weight for volume
Y	C or T

Chapter One: Introduction

1.0 '*Septoria*' diseases

Septoria is the common genus name for more than 1,000 species of fungi most of which are plant pathogens. Approximately 100 species are pathogenic on cereals and grasses. Many are economically important on crops other than cereals (Eyal *et al.*, 1987). There are two major *Septoria* diseases that infect wheat in many parts of the world. These are leaf blotch of wheat caused by the fungus *Septoria tritici* (sexual state: *Mycosphaerella graminicola*) and glume blotch of wheat caused by the fungus *Stagonospora nodorum* (syn. *Septoria nodorum*, sexual state: *Phaeosphaeria nodorum*, syn. *Leptosphaeria nodorum* (Cunfer, 1997)). Also able to infect wheat and barley, is *Stagonospora avenae* f.sp.*triticea* (syn. *Septoria avenae* f.sp. *triticea*, sexual state: *Phaeosphaeria avenaria* f.sp. *triticea*). Together *S.nodorum* and *S.tritici* have often been termed the *Septoria* disease complex of cereals. This is because they both cause similar symptoms on leaves and often occur together on the same leaf, where they produce similar pycnidia. This makes field identification, without confirmation in the laboratory difficult, if not impossible. Both are economically important pathogens but this work will only focus on *S.nodorum* as until recently this was the more economically important and better characterised of the two.

1.0.1 Disease symptoms

Stagonospora nodorum can infect seedlings, resulting in twisted, distorted and stunted plants. On mature plants the disease affects all aerial parts, the leaves showing symptoms about 10 days after infection. The leaf lesions are up to 1cm long, yellow at first but then small necrotic spots appear which later become golden brown and badly infected plants often appear ginger in colour. The shape of lesions is variable but is often elliptical and surrounded by a darker, slightly purplish margin. The leaf spots eventually coalesce, resulting in death of the leaves, after which pycnidia occur abundantly in the dead tissue. The nodes of

the culm and rachis are also often attacked and become purplish-black and also bear pycnidia. Leaf infections have been shown to cause reductions in yield, however the greatest losses occur from infection of the heads, resulting in drastic reductions in grain size. Such infection of the glumes can be extensive, the dark, purplish-brown lesions spreading downwards from the tip. Pycnidia are produced in these lesions and in cases of severe infection, on almost all of the glumes. Symptom expression will depend on isolate, host cultivar and environment (Karjalainen, 1985).



Fig.1.1. Wheat leaves infected with *Stagonospora nodorum*

1.0.2 Disease cycle

Stagonospora nodorum overwinters as dormant mycelium, pycnidia and pseudothecia on seed, stubble, debris, autumn sown crops and volunteers. An important source of primary inoculum in the autumn or spring are wind-borne ascospores (sexual spores) released from pseudothecia, particularly because ascospores can germinate and infect when temperatures are lower than 5°C, while pycnidiospores (asexual spores) require temperatures above 5°C to infect leaves (Rapilly and Skajennikoff, 1974).

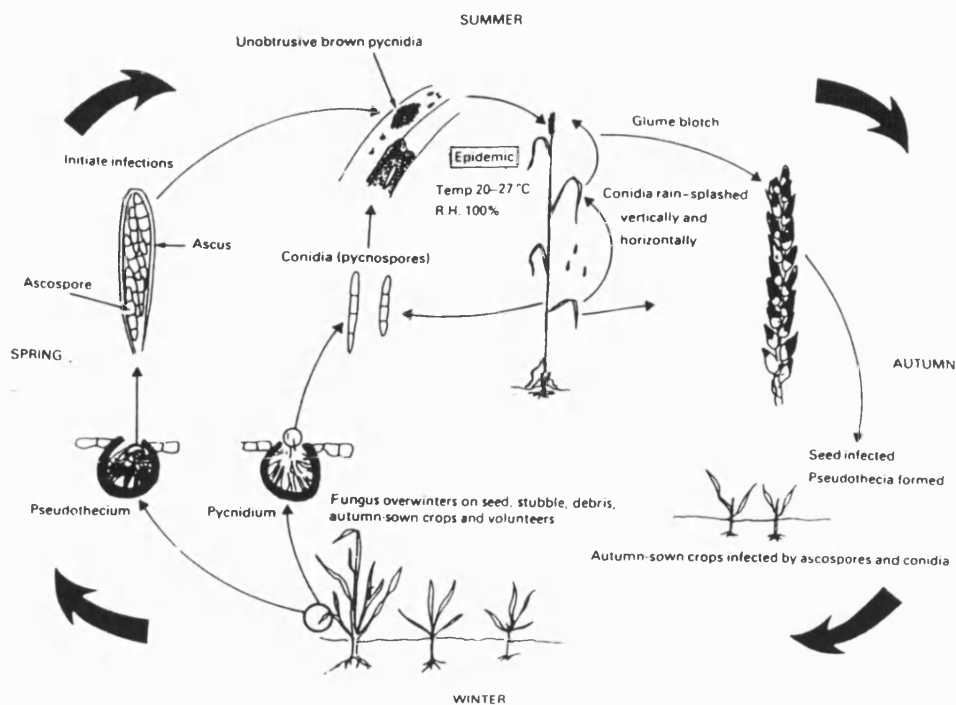


Fig. 1.2. Disease cycle of leaf and glume blotch (*Stagonospora nodorum*). From Parry (1990).

This means that young plants are more likely to be infected with ascospores in late autumn and early spring. Asexual pycnidiospores are produced in pycnidia as temperatures rise and humidity increases and are also an important inoculum source. New pycnidia form abundantly in debris if it is alternately wet and dry and pycnidiospores are released over long periods of up to several months, in gelatinous spore masses known as cirri. The presence of the surrounding cirrus gel prevents germination of the spores and prevents desiccation; 40% of the dry weight of the gel comprises proteins and carbohydrate (the remaining 60% v/v is spores) (Fournet, 1969). Rain is essential for spore release and dispersal and is assisted by accompanying air turbulence or winds. Dilution of the spores by rain also dilutes out the cirrus gel and thus the germination inhibitor. In fact, dilute extracts of cirri stimulate germination of pycnidiospores (Rapilly and Skajenikoff, 1974). Splash-dispersal results in pycnidiospore distribution vertically up the plant and horizontally from plant to plant. Under optimal conditions for spore production and germination i.e. temperatures of 20-27 °C

together with 100% humidity, the disease cycle can be completed in 10-14 days. At the end of the season, infected leaves and ears can provide spores to initiate infection in early autumn sown crops and volunteers or spores may remain dormant over the winter. Infected ears provide another inoculum source in the form of infected seed on which spores can survive for at least 2 years. Seedborne infection can result in infection of the growing coleoptile on which pycnidia form and provide a secondary inoculum for infection of the leaves (Parry, 1990).

Asexual spores have largely been thought of as the most important inoculum source at all stages of the disease cycle and this is probably true on small spatial scales of 1 to 2m. It is clear that the dispersal of pycnidiospores by rain splash will limit their dispersal within a field and preclude long distance travel. This is supported by genotype studies demonstrating little variation within field populations. The same genotypes were common between lesions on the same leaf but rare when sampling locations were separated by 10m or greater in a field (Keller *et al.*, 1997). If asexual spread was most common there should be a limited number of genotypes present in field populations; however this was not the case. Therefore, windborne ascospores are likely to be important in the population biology of *S.nodorum* on a regional scale. Sexual reproduction would explain the high genotype variation observed in populations of *S.nodorum* within a field and even within a single leaf; in one case seven different genotypes were collected from a single leaf (Keller *et al.*, 1997). Sexual reproduction may also explain why field populations are so similar on a regional scale because spread of ascospores among fields would unify populations on a regional basis into a large metapopulation. The importance of the sexual cycle in disease has previously been underestimated (Eyal *et al.*, 1987) but its relevance to disease management must now be considered. New ascospore immigrants may be capable of overcoming local plant resistance and through sexual recombination pass on that ability to the resident population of *S.nodorum*.

1.0.3 Host Specificity

Stagonospora nodorum is the causal agent of leaf and glume blotch of wheat (Weber, 1922), first described by Berkeley in 1885. It also produces a similar disease on barley, although it is less frequent and far less damaging (Holmes and Colhoun, 1970). Most isolates of *S.nodorum* are specialised to either wheat or barley (Cunfer and Youmans, 1983). However, some work suggested that these adaptations could be changed by passing through the non-adapted host (Rufty *et al.* 1981). This work was contradicted by two independent investigations by Osbourn *et al.*(1986,1987) and Bereck *et al.* (1990); both found that serial passaging through non-hosts failed to alter individual genotype's host adaptation. Osbourn *et al.* (1986, 1987) followed isolates using genetic markers and showed that apparent changes in adaptation were due to selection of a virulent contaminant rather than real adaptations. Three cycles of passage of wheat isolates through barley increased virulence towards barley but also to wheat (Bereck *et al.* 1990). Further studies on host adaptation, cultural, physiological and biochemical characters have strengthened the view that genetically distinct populations of wheat (W) and barley (B) adapted types exist (Newton and Caten, 1991). None of the characters assessed were causally associated with adaptation to W or B-type. Genetic studies have since shown that there is considerable divergence at the DNA level between all strains but this is not related to B or W-types (Cooley and Caten, 1991). Karyotype studies showed that the genome contains around 30Mb of DNA organised into 14-19 chromosomes ranging from 0.4 to 3.5 Mb in size. There was no correlation between strain karyotype and host adaptation.

S.nodorum is also capable of infecting perennial grasses which may allow the pathogen to survive between wheat crops. To test this theory, isolates from 13 perennial grasses were assessed for their ability to cause disease on wheat. Some were capable of causing large lesions, although most only produced small lesions (Krupinsky, 1997a). Successive passage of these grass isolates through wheat failed to increase their aggressiveness or adapt them to wheat, suggesting that

generally these isolates are unlikely to cause severe symptoms on wheat (Krupinsky, 1997b). They still remain a potential source of inoculum and also represent a possible source of genetic variability.

1.0.4 Geographic distribution and losses

Stagonospora nodorum has been reported to occur in many parts of Europe, North and South America, Africa, Asia, Australia and New Zealand (King *et al.*, 1983). The shrivelled grain resulting from infection reduces grain weight and the occurrence of the disease late in the season can cause losses of up to 50%, although annual losses in the UK probably do not exceed 10% (Parry, 1990). The disease increased in importance from 1975-1985 reflecting increased wheat growing in areas with cool damp summers and mild winters. The increased use of dwarf varieties has favoured disease by reducing the distance that spores have to travel by rain splash and hence more of the plant becomes infected. Non ploughing techniques have also increased disease incidence because failure to remove crop debris provides another inoculum source. From 1978-1982 and again in 1986 *S.nodorum* was the most important foliar disease of winter wheat in the UK. In 1985, 1987 and 1988 *Septoria tritici* became the more important pathogen (Polley and Thomas, 1991). *Septoria tritici* disease intensity used to decline following winter and early spring development. Now however it frequently develops rapidly in the summer in Western Europe. This trend appears to coincide with a decline in the levels of *S.nodorum*. This has coincided with changes in crop varieties, increased use of wheat in crop rotation and the use of MBC (benzimidazole) fungicides (Polley and Thomas, 1991). MBC fungicides favour *Septoria tritici* because of the occurrence of resistant strains, first reported in 1984. ADAS surveys of cereal crops in England and Wales show that for the four year period 1986-1989 *Septoria tritici* was the most damaging disease of winter wheat, causing average annual losses of 2% (Cook *et al.* 1991). In contrast *S.nodorum* was the fourth most damaging disease behind eyespot and mildew, causing 0.9% loss. Average annual loss figures for 1981-1988 are 1.9% and 1.7% for *S.tritici* and *S.nodorum* respectively (Oerke *et al.*

1994). The proportions of disease caused by *S.nodorum* and *Septoria tritici* vary greatly from year to year, and whilst currently *Septoria tritici* is the more important pathogen, *S.nodorum* still poses a threat as an important disease of wheat, as has been demonstrated in the past.

1.0.5 Identification and diagnosis

Field identification in the past has been based on symptoms. Subsequent identification has traditionally been by conidial morphology. The conidia have 1-3 septa and are usually no longer than 25 µm, however these features can be affected by environment (King *et al.* 1983). More accurate verification to field diagnosis can be by ELISA (Lagerberg, 1996) or molecular methods such as PCR which are being developed (Beck and Lignon, 1995), to differentiate between *S.nodorum* and *Septoria tritici* earlier in infection.

1.0.6 Host colonisation

Septoria tritici penetrates through stomata or directly through the cuticle and cell walls of the epidermis and quickly assumes intercellular mesophyll growth (Eyal *et al.*, 1987). In contrast, *S.nodorum* penetrates directly through the intact cuticle and grows subcuticularly for a while before penetrating epidermal cell walls and eventually commencing intercellular growth in the mesophyll. Extensive epiphytic growth by *S.nodorum* is accompanied by a large number of unsuccessful penetrations; even on a susceptible host up to 90% of penetrations can fail (Bird and Ride, 1981). This low infection efficiency is compensated for by the large numbers of spores produced from pycnidia. Ultrastructural studies have provided useful information on the penetration and growth of *S.nodorum* within the plant and host resistance responses. These aspects will be detailed below.

1.0.6.1 Adhesion and germination

There have been no direct studies on adhesion of *S.nodorum* spores to the leaf surface. However, an amorphous material surrounding germ tubes which appeared to effect attachment to the host surface has been observed (Karjalainen and Lounatmaa, 1986). A large amount of mucilage covering infection structures of the isolate used in this study has also been observed (Rundle, 1997) (Fig 1.3).



Fig.1.3. Growth of *S.nodorum* on the wheat leaf surface 48h after inoculation. Note the mucilage covering hyphae. Cryo-scanning electron microscopy (x2,300).

In addition to a role in adhesion this extensive mucilage may provide *S.nodorum* with nutrients to support its extensive epiphytic growth (Baker and Smith, 1978). Spores of *S.nodorum* produced in pycnidia are contained within a cirrus gel and it is possible that any mucilage remaining associated with spores following dilution and dispersal may be involved in attachment to the host.

Initial attachment of fungal propagules to the host plant surface is essential to the successful establishment of a pathogen (Nicholson and Epstein, 1991). Adhesion will prevent displacement of spores by wind or rain and may play a role in host

recognition. Attachment, formerly thought of as purely a chance event, is now known to be caused by active secretion of adhesive materials and may involve the secretion of fluids and enzymes that alter the host surface to allow adhesion. Secreted adhesives have so far been found to be polysaccharides, proteins or glycoproteins and may also be associated with germ tubes and appressoria. Examples of these adhesives have been found associated with the conidia of *Magnaporthe grisea* (Hamer *et al.* 1988), *Colletotrichum graminicola* (Nicholson and Kunoch, 1994) and *Phytophthora megasperma* f.sp.*glycinea*. In the latter case the nature of the glycoproteins involved in adhesion to hydrophobic and hydrophilic surfaces differed (Hollenstein *et al.*, 1995).

Interestingly, cutinase activity has been found associated with the spores of *S.nodorum* produced *in vitro* and could possibly play a role in attachment, or penetration (Dewey *et al.*, 1997). This is supported by apparent removal of wax from under conidia and hyphae of *S.nodorum* on the leaf surface (Baker and Smith, 1978; Zinkernagel *et al.*, 1988). Enzymes such as cutinase may be required to alter the host surface in order for adhesion to take place. Uredospores of the obligate fungus *Uromyces viciae-fabae* form an adhesion pad on contact with the leaf surface and at the same time release a cutinase which assists in adhesion of the spore to the host (Deising *et al.*, 1992). Degradation of host cuticular waxes occurs after conidia of *Erysiphe graminis* f.sp.*hordei* land on the leaf (Pasholati, 1992) coincident with cutinase release (Nicholson *et al.*, 1988). The ability to degrade the host cuticle may also contribute to determining host specificity (Staub *et al.* 1974). *E.graminis* is capable of eroding the surface of both barley and cucumber while *E.cichoracearum* only erodes the cuticle of cucumber.

Following attachment to the host, *S.nodorum* spores germinate under conditions of high humidity and suitable temperatures from as early as 2h after inoculation with 90% germination by 6-8h and 100% by 12h (Bird and Ride, 1981). During the following 48h extensive hyphal growth ramifies irregularly over the leaf surface (Baker and Smith, 1978).

1.0.6.2 Host penetration

Penetration of the host by a plant pathogen can be achieved by a number of different means. Penetration can occur through natural openings such as stomata or wounds or directly through the cuticle (Dean, 1997). The latter may be achieved by enzymic or mechanical means or by a combination of the two. There is no direct evidence whether mechanical or enzymic methods are employed by *S.nodorum* in effecting penetration. During the first 48 h after inoculation appressoria form at the junction of cell and walls (Baker and Smith, 1978) and four to five appressoria per spore were observed by Bird and Ride (1981). In contrast, in a study by Zinkernagel *et al.* (1988) although appressoria were observed, the favoured route of entry by *S.nodorum* was direct penetration of the cuticle by germ tubes. Different modes of penetration within the same species is not uncommon, for example, *Rhizoctonia solani* penetrates its host directly, from an infection cushion or even occasionally through stomata (Murray, 1982). Appressoria are structures employed by some fungal pathogens to press against and attach to the plant surface in preparation for infection (for review see Dean, 1997). The best characterised appressoria are those of *M.grisea* which is believed to penetrate mechanically the rice leaf (Howard *et al.*, 1991). Appressoria of *M.grisea* are very characteristic with their thick multilayered and highly melanised cell wall. Enormous turgor pressure builds up within the appressorium and is thought to force the penetration peg through the leaf. Melanin deficient mutants make nonfunctional appressoria and are consequently nonpathogenic (Howard and Ferrari, 1989).

The rare occurrence of appressoria and the lack of a distinct, melanised appressorium structure for the isolate of *S.nodorum* used in this study, was observed by Rundle (1997) (Fig 1.4).

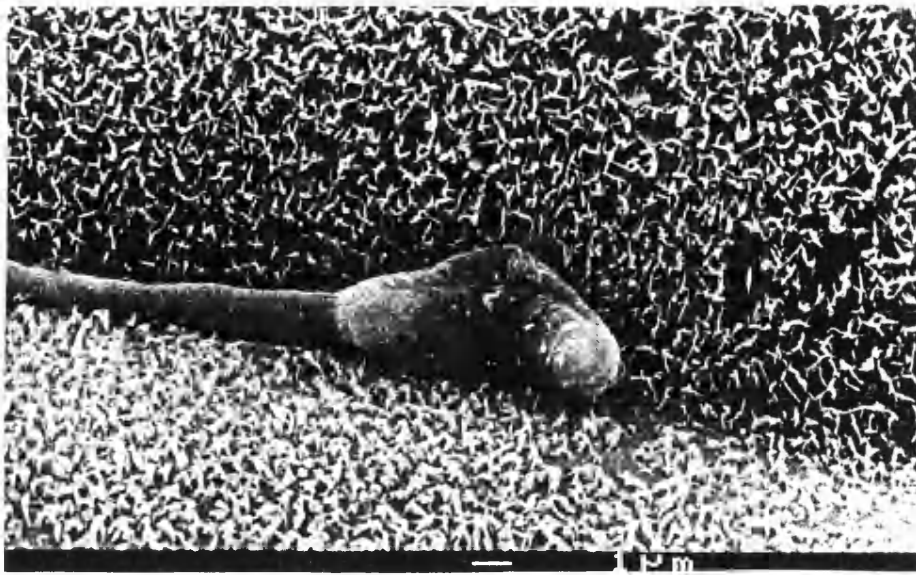


Fig.1.4. An appressorium produced by *S.nodorum* on a wheat leaf after 48h. Cryo-scanning electron microscopy (x4,500).

The absence of defined, melanised appressorium structure combined with the ability of *S.nodorum* to penetrate the cuticle directly suggests that enzymic penetration is likely to be more important than mechanical. Electron microscopy studies by Karjalainen and Lounatmaa (1986) showed the host cell wall beneath the appressorium and other parts of the cell wall in contact with the penetration peg were more diffuse than the normal wall, indicating a role for cell wall degrading enzymes in penetration. Indeed, the cell wall degrading enzymes xylanase, polygalacturonase and cellulase have been associated with *S.nodorum* infection of wheat (Magro, 1984). Baker and Smith (1978) observed the disappearance of the wax layer of the cuticle under hyphae and appressoria of *S.nodorum*. Excretions from conidia and hyphae also appeared to be lysing the cuticle in the study by Zinkernagel *et al.* (1988) where direct penetration of the host was observed. Both these studies suggest a possible role for cutinase in penetration. Cutinase activity has been detected associated with spores produced *in vitro* (Dewey *et al.*, 1997), although its presence *in vivo* has yet to be demonstrated.

Cutinases may be important in host penetration by plant pathogens. Most notably, the insertion of the cutinase gene from *F.solani* f.sp.*pisi* into the wound pathogen of papaya fruits, a *Mycosphaerella* sp, enabled this fungus to infect the host through the intact cuticle (Dickman *et al.*, 1989). This new ability to penetrate the cuticle could be inhibited by antibodies against *Fusarium* cutinase.

1.1.6.3 Host colonisation and resistance

Bird and Ride (1981) first observed penetration of the host by *S.nodorum* 10h post inoculation, with only 1% and 5% of appressoria giving rise to successful penetrations in resistant and susceptible varieties respectively. Most of the successful penetrations occurred in the thin walled cells above the sclerenchyma. Many of the unsuccessful penetrations were associated with deposition of papillae (new wall-like material) and alterations in the upper epidermal walls and adjacent lateral walls. Histochemical tests and fluorescence microscopy showed these to be lignified papillae. Many penetrations appeared to involve a subcuticular or lateral growth within the wall rather than direct penetration of epidermal cells. Zinkernagel *et al.* (1988) observed a similar extensive mycelial growth beneath the cuticle from which penetration of the epidermal cells occurred. In contrast, in a study by Karjalainen and Lounatmaa (1986), the major route of penetration appeared to be direct into the lumen of the cell and only some lateral subcuticular growth was observed. These contradictions may reflect differences in host susceptibility or in fungal strains. It appears possible that *S.nodorum* can employ either mode of penetration, although more studies have revealed subcuticular growth within the wall rather than direct penetration.

Following penetration, epidermal cells collapse and lignify and hyphae grow slowly producing a sparse intercellular mycelium within the mesophyll. Mesophyll cells in advance or associated with fungal hyphae become misshapen, the cell walls lignify, the cytoplasm degenerates and lignifies and cells frequently collapse (Bird and Ride, 1981, Zinkernagel *et al.*, 1988). In these studies hyphae did not pass the bundle sheath and enter the vascular tissues, probably due to the natural lignification of the bundle sheath walls. As with other necrotrophs

(Cooper, 1981), marked changes in host ultrastructure such as chloroplast disintegration and chlorophyll breakdown occur soon after infection (Karjalainen, 1985).

Resistance of wheat to *S.nodorum* is polygenic, suggesting a number of mechanisms may be involved, possibly acting at different stages of infection. One suggested resistance response that has been studied extensively is the lignification observed in response to penetration and growth of *S.nodorum* through the plant. No effects of variety resistance on germination or number of appressoria produced have been observed. However, germ tubes on resistant varieties may be shorter (Bird and Ride, 1981). The mechanism for this difference is unknown although such differences were abolished in the presence of cyclohexamide, suggesting it is dependent on induced protein synthesis. In the same study many penetration events failed and this has been frequently seen in other studies of *S.nodorum* infection. These are in part at least, associated with papillae formation (new wall-like material), alterations in the upper epidermal walls (haloes) and adjacent lateral walls. Bird and Ride (1981) showed that these structures were lignified and occurred in both susceptible and resistant varieties. Lignification of invaded tissues never totally contained the hyphae and there was no correlation between lignification and host resistance. Lignification appeared to reflect more the extent of fungal colonisation than resistance. It may be that lignification acts as a general resistance response in all varieties reducing fungal growth and colonisation. In the case of the more resistant varieties it may be other factors that reduce fungal growth, possibly then allowing lignification to be more effective. Lignification as a general defence response may act as a mechanical barrier to hyphae, protecting the host cell wall from enzymic attack or restricting the diffusion of water, nutrients and toxins. Baker and Smith (1978) however, failed to observe any induced lignin formation in resistant or susceptible varieties. They also observed reduced colonisation of resistant varieties, suggesting that lignin is not an essential component of resistance. Studies on the incompatible reaction between a wheat-adapted isolate of *S.nodorum* and barley found growth restricted by the deposition of papillae (Keon and Hargreaves, 1984). However, while these appear to have some similar

components to papillae observed in wheat they are thought to be composed of glycoproteins more than lignin (Hargreaves and Keon, 1986). More recent work on defence genes induced in barley during this interaction have revealed genes encoding β 1,3-1,4 glucanase, Bowman-Birk type proteinase inhibitor, a PR-1 protein, leaf-specific thionin, peroxidase and a 3-hydroxy-3methylglutaryl-coenzyme A reductase (Stevens *et al.*, 1996). This work suggests that a number of different cellular processes are occurring during this resistance reaction, aside from papillae deposition.

1.0.7 Control

Control of *S.nodorum* can be achieved with a combination of cultural practices, resistance breeding and fungicide application. Cultural control methods include crop rotation and ensuring disposal of crop debris, formerly by burning but now by ploughing in. There are no truly resistant wheat varieties, while some are merely tolerant or moderately resistant. Resistance is under polygenic control and breeding problems also result from the fact that head and leaf resistance are inherited independently (Fried and Meister, 1987). Fungicides are routinely used to control *S.nodorum*; protective seed treatments and protective and curative foliar sprays are applied in response to symptoms on young tissue and/or conducive weather conditions, to protect the flag leaf and ear from infection to ensure good grain filling. A high risk period for *S.nodorum* has been defined as three consecutive rain days in which at least 10mm have fallen in the two weeks previous to flag leaf emergence (growth stage 37).

Because of the rate of development of resistance to current fungicides, continual development of novel fungicides is required to ensure effective control of economically important plant pathogenic fungi such as *S.nodorum*.

1.1 Rational Design of Fungicides

1.1.1 Fungicide research and development

Plant pathogens are estimated to cause yield reductions of almost 20% in the principal food and cash crops worldwide (Oerke *et al.*, 1994). Attempts to control these diseases are based on a combination of disease-tolerant cultivars, crop rotation, sanitation practices and fungicides. The predicted growth of the world's population from 5.7 billion in 1993 to approximately 8 billion by 2020 (Knight *et al.*, 1997) means that there will be an increased pressure on food production. Increased yields will have to be achieved by optimising inputs for staple food crops such as cereals, rice and maize. In the absence of durable plant resistance to many key pathogens, progress is likely to require the continued use of fungicides and hence for the agrochemical industry there is a need to continue to develop novel, durable fungicides.

Disease control using chemicals systematically began during the 1850s when Bordeaux Mixture was introduced to control downy mildew (*Plasmopara viticola*) in French vineyards (Brent, 1985). In the first half of this century, protection of many crops became possible with the advent of organic fungicides such as the dithiocarbamates. These were surface protectants that do not penetrate plant tissue so to be effective they must be applied in advance of infection and repeated applications are required to protect new tissue. In the 1960s the development of systemic products such as the benzimidazoles and 2-aminopyrimidines that are able to penetrate plant tissue and deliver curative properties permitted a more flexible application regime (De Waard *et al.*, 1993). Some of the more recent compounds are not fungitoxic but still control plant diseases by interference with processes involved in fungal penetration into the plant or by enhancing host-plant resistance. For example tricyclazole leads to the malfunctioning of appressoria and probenazole triggers plant defence mechanisms (Sisler, 1986; Sekizawa and Mase, 1981). There are now 113 active ingredients registered as fungicides worldwide (Knight *et al.*, 1997) but new

antifungal chemicals are still needed to deliver improved yield and quality. New fungicides are also required, because of government legislation and public pressure, to be used at low rates, be environmentally benign, non-persistent, and have a low toxicity to humans and wildlife. Improved fungicides are also needed to combat diseases, such as vascular wilts and anthracnoses that are poorly controlled by current products. Also required is a continuing development of fungicides with novel modes of action to combat pathogens that have developed resistance or reduced sensitivity to existing antifungal compounds (Staub, 1991). An estimated \$570 million is now spent annually on research and development by the leading 15 agrochemical companies (Knight *et al.*, 1997).

A number of approaches to fungicide development are now being taken in combination with the traditional method of empirical chemical screening (Goosey, 1992). One such approach is screening natural products such as antibiotics and secondary metabolites found in fungi, bacteria and higher plants for anti-fungal activity. One successful product generated by this approach is azoxystrobin, a broad spectrum fungicide which controls major economic pathogens from all four taxonomic groups of fungi (Godwin *et al.*, 1992). This chemical is a synthetic analogue of a group of natural products, the strobilurins found in certain basidiomycetes. The anti-fungal activity is a result of inhibition of mitochondrial respiration. Identification of compounds such as these and determination of their mode of action opens up opportunities for the design of other chemicals directed at those sites. This has also been true for other traditional compounds found by random screening approaches such as the ergosterol biosynthesis inhibitors. The discovery in 1972 that triarimol, a highly active systemic plant fungicide, inhibited the synthesis of ergosterol (Ragsdale and Sisler, 1972) enabled other chemicals directed at this target site to be generated. Since then over 20 14-demethylase inhibitors of sterol biosynthesis have been developed as agricultural fungicides and this group currently accounts for 24% of global fungicide sales (Knight *et al.*, 1997).

Identification of a target site that synthetic compounds can then be directed against has in the past been known as rational fungicide design but has more recently been termed biorational design.

1.1.2 Biorational fungicide design

Biorational fungicide design requires the identification of a suitable, novel biochemical target in the fungus that a fungicide can be directed against. The starting point therefore is to identify key processes in the life cycle of the pathogen essential for survival; spread or pathogenicity. Current fungicide targets with this type of mode of action include ergosterol, methionine and melanin biosynthesis inhibitors (Berg and Tiemann, 1995). Other possibilities include targets in cell wall biosynthesis, such as glucan (Current *et al.*, 1995) and chitin synthesis (Munro and Gow, 1995), sporulation, respiration, differentiation, protein secretion and novel pathways specific to fungi such as lysine biosynthesis (Timberlake, 1995). Other alternatives exist in processes essential for pathogenicity of the fungus. Pathogenicity factors are broadly defined as factors necessary for disease development but not for normal growth on artificial media (Oliver and Osbourn, 1995). Most of the 100,000 known species of fungi are strictly saprophytic and degrade dead organic materials for their source of nutrients. Only 8000 can cause disease on one or more plant species and only 100 are pathogenic to humans or animals (Agrios, 1988). What then distinguishes this small minority of pathogenic fungi from the majority of saprophytic fungi? Are there major genetic differences between the two groups? There are three possible answers: Saprophytic and pathogenic fungi both share the same set of genes, but some genes are differentially regulated; pathogenic fungi possess unique genes that enable them to be pathogenic; or a combination of these two possibilities (Schafer, 1994). Factors likely to be important in establishing disease are: attachment to the plant surface, germination and formation of infection structures, penetration of the host and colonisation of the host tissue. Also, at every stage of infection the plant's defence responses must be avoided, overcome or suppressed. With these different processes in mind

there are a number of 'logical' pathogenicity factors; cell wall degrading enzymes (CWDE) to overcome physical barriers, toxin production to debilitate or kill host cells, the ability to detoxify pre-formed plant inhibitors and phytoalexins and for some bacteria, the ability to produce extracellular polysaccharides to avoid recognition (Oliver and Osbourn, 1995; Daniels *et al.*, 1988). The importance of these pathogenicity factors will vary between different pathogen-plant interactions, depending on factors such as mode of penetration and growth within the plant, whether the pathogen is necrotrophic or biotrophic, what pre-formed inhibitors are present in the plant and the plant's defence responses.

Other studies are also required to take new approaches to the identification of pathogenicity genes, not previously studied as 'logical' pathogenicity determinants. Such techniques include random insertional mutagenesis or differential screening (Oliver and Osbourn, 1995; Van Etten *et al.*, 1994). These methods have already been successful in identifying new pathogenicity genes. Transposon mutagenesis combined with genetic complementation has identified the *hrp* gene cluster responsible for elicitation of the hypersensitive response elicited by *Erwinia amylovora* (Beer *et al.*, 1989). Differential cDNA cloning was used to identify pathogenicity genes that are expressed during growth of *Magnaporthe grisea* in planta. Several genes were identified by this screen and one, *MPG1* encodes a hydrophobin (Talbot *et al.*, 1996). Disruption of the *MPG1* gene reduced disease by 80%, apparently reducing the ability of *M.grisea* to differentiate appressoria. Hydrophobic rodlet proteins are thought to be important in the attachment of some fungi (Dean, 1997), however this is probably not their role in *M.grisea*. Instead they may play a role in surface recognition (Beckerman and Ebbole, 1996).

A useful technique that causes and tags a mutation in one step is restriction enzyme-mediated integration or REMI (Kuspa and Loomis, 1992; Schiestl and Petes, 1991). Transformation is carried out with a non-homologous vector in the presence of a restriction enzyme that linearises the vector. This treatment stimulates integration of the vector into the cut sites within the chromosomes and

increases transformation efficiency (Shi *et al.*, 1995). This results in a population of transformants with insertions distributed at random throughout the genome, these can then be screened for mutant phenotypes, including ones that affect pathogenicity (Lu *et al.*, 1994; Shi *et al.*, 1995). Sequence of the inactivated gene can be retrieved by digesting the mutant DNA with an enzyme that cuts outside the integrated plasmid, followed by re-circularisation and transformation into *E.coli* under ampicillin selection. This technique has been successfully used to isolate toxin deficient mutants of *Alternaria alternata* and *Cochliobolus heterostrophus* (Akamatsu *et al.*, 1997; Lu *et al.*, 1994) and to generate mutants of *M.grisea* with altered pathogenicity (Sweigard *et al.*, 1998; Balhadere *et al.*, 1999).

Genomics is another tool recently employed by agrochemical companies in the identification of novel fungicide targets (Schmid, 1998). The development of technologies and strategies for efficient genomic sequencing has resulted in the complete sequencing of several microbial genomes, including *E.coli* and *S.cerevisiae* and the on-going sequencing of the larger genomes of *Arabidopsis thaliana* and *Homo sapiens* (Fraser and Fleischmann, 1997). These sequence data combined with publicly and commercially available sequences from other organisms allows, with the use of computer based analysis programmes, the spectrum and suitability of a target to be identified. Firstly, essential genes in the organism of interest can be selected by comparison to other more thoroughly studied species. Homology searches can then be used to select genes present in the target organism (s) that are not present in plants or humans. Obviously as the function of more of the sequenced ORFs becomes known, more potential targets may be identified.

Having identified a possible target it is then important to validate it as a potential fungicide target. Biochemical and immunocytochemical and classical genetical approaches have implicated a number of factors in pathogenicity but have often been unable to prove unequivocally that they are required for the ability to cause disease. Classical genetics has been successful in some cases, for example in the

identification of the single gene locus, *Tox1* which controls the production of T-toxin in *Cochliobolus heterostrophus*. Production of this toxin renders the fungus highly virulent toward Texas male sterile (T) cytoplasm corn and when race T is crossed with a naturally occurring non-toxin producing strain, race O, half the progeny produce toxin and half do not (Yoder and Gracen, 1975). This defined *Tox1* as a single locus determining whether or not T-toxin is produced and whether the fungus is highly virulent. Single loci have also been identified by classical genetics as being responsible for the production of Victorin by *C.victoriae* and HC-toxin by *C.carbonum* (Walton, 1996).

Where there is circumstantial evidence for the role of a pathogenicity factor in disease, its potential as a fungicide target can be tested by creating mutants in the gene encoding the pathogenicity factor of interest. Mutants can be generated randomly by chemicals or radiation followed by analysis for loss of gene function e.g. enzyme production. However, mutants created by these methods may harbour multiple mutations and it may be difficult to identify the affected gene since low transformation frequency in many fungi prevents cloning by self complementation (Caten and Holloman, 1995).

The development of DNA-mediated transformation techniques for phytopathogenic fungi have enabled the direct testing of the importance of putative pathogenicity determinants. Gene disruption by transformation with cloned DNA sequences has enabled the construction of specific, defined mutants which are defective only in the gene of interest (Fincham, 1986). This should allow the exact role of the gene in pathogenesis to be determined. This technique is not applicable to diploid or polyploid fungi such as potato late blight (*Phytophthora infestans*) because homologous recombination of all copies of the gene must be achieved. Similarly, it is currently impossible to transform obligate fungi such as the rusts and mildews. A number of fungi therefore exist in which this approach to identifying gene function is not possible.

Before this approach can be used at least some of the gene of interest must be cloned and sequenced. There are a number of approaches to isolating genes from fungi (Timberlake, 1991) and the principal methods are listed here: (1) Heterologous screening of a genomic library with previously cloned genes of related function from other organisms; (2) Homologous screening with oligonucleotide probes based on N-terminal or internal protein sequence, generated from the target protein itself; (3) Differential screening; (4) Complementation of an existing biochemical mutant with the wild type gene. This has been particularly useful in species such as *Aspergillus nidulans* and *Neurospora crassa* where transformation frequency is high and a large number of biochemical mutant strains are available; (5) Heterologous complementation of these mutants with DNA from other fungal species; (6) Chromosomal walking for the isolation of closely linked genes; (7). Detection of the desired clone in a cDNA library by antibody recognition; (8) Isolation of the gene by degenerate PCR using primers based on consensus sequences.

Targeted gene disruption is brought about through the integration of exogenous DNA into the target gene through homologous recombination (Caten and Holloman, 1995). To achieve this two methods have been employed. In the first, a cloned internal fragment of the gene of interest is inserted into a simple vector which carries a selectable marker, such as hygromycin resistance. Once transformed this vector will, in a proportion of cases, integrate into the target gene through homologous recombination, resulting in the formation of two truncated versions of the target gene separated by the vector sequence. Sometimes the first part of the disrupted gene, which is only truncated at the 3' end may still produce an active protein. Loss of function therefore must be confirmed by assay. In the second method, a clone carrying the target gene and flanking 5' and 3' regions is engineered *in vitro* so that a selectable marker is present in the middle of the coding region and some or all of the coding region is removed. When this construct is transformed, either as a linear fragment or as part of a circular vector, integration will occur in a proportion of transformants at

the homologous site by a double cross over. This will replace the functional target gene with the inactivated one.

These procedures were first developed in *S.cerevisiae* where the efficiency of homologous recombination is high (Rothstein, 1983). In filamentous fungi it appears that heterologous integrations occur frequently, and only a proportion of transformants show the desired phenotypic effect (Fincham, 1989). In these cases detection of the desired transformants can be difficult, especially if the phenotype is not easily assayable.

S.nodorum has been successfully transformed with dominant selectable markers for hygromycin B and benomyl resistance (Cooley *et al.*, 1988, 1991). Despite the use in the latter case of the homologous β -tubulin gene, ectopic integrations predominated. In contrast, no ectopic integrations were observed following homologous transformation of nitrate reductase mutants with the wild-type nitrate reductase gene (Cutler *et al.*, 1998). The cloning of this gene also provides another selectable marker for transformation. However, in all cases transformation efficiencies were low. Subsequently the nitrate reductase gene has been disrupted by one step gene replacement (Kirsty Howard personal communication) as has the ornithine decarboxylase gene (Andy Bailey personal communication).

One disadvantage of using targeted gene disruption for fungicide target validation is that removal of the gene completely eliminates the gene product. In the field situation perhaps only around 70% inhibition can be achieved by a chemical, hence other studies are then required to determine if this partial reduction in activity is sufficient to prevent disease. An alternative approach to address this problem is RNA antisense technology because this frequently does not completely inhibit the target gene (Mol *et al.*, 1990). This technology has previously been used in the validation of herbicide targets, such as acetolactate synthase, the first enzyme in the biosynthetic pathway of leucine, valine and isoleucine (Hofgen *et al.*, 1995). In this case an 80% inhibition of the enzyme

rendered the plants almost inviable without amino acid supplementation, confirming this as a valid target. This technology could now be applied to the validation of fungicide targets.

Once a pathogenicity determinant has been validated, provided it has an assayable activity, the aim of the agrochemical company is to overexpress the gene in a recombinant system. This will serve to supply a protein source for use in high throughput screens in a microtitre plate format. These screens allow a larger number of chemicals (>10,000 per week) to be screened against a specific target of interest rather than the traditional screens of relatively low numbers (10,000 per annum) against the whole organism (Major, 1995).

Many of these studies on finding novel fungicide targets are being performed on *Stagonospora nodorum* for a number of reasons. It is an ascomycete, a group that contains a number of other important plant pathogens; it is a pathogen of wheat, an important cereal crop; it is easy to culture and screen *in vivo* with detached leaf assays (Benedikz *et al.*, 1981); and finally a method for transforming this fungus (to validate targets by gene disruption) has been established and gene disruptions successfully achieved.

1.2. Pathogenicity Factors - Fungicide Targets?

The term pathogenicity factor has been widely used to describe anything required by a fungus to cause disease on a plant. Often no distinction has been made between whether these are pathogenicity or virulence determinants.

Pathogenicity determinants are generally considered as an absolute requirement for a pathogen to cause disease on a plant, for example detoxification of pre-formed antifungals, such as saponins. In contrast, virulence factors may be considered as not critical for causing disease but just one weapon that if lost might reduce disease severity, e.g. host non-selective toxins. Because these two types have so frequently been grouped together they will be considered together here and given the general term pathogenicity factors. However the difference

can be clear cut and would influence decisions as to the selection of potential 'fungicide' targets.

Nature of Process	Plant Pathogen	Molecular determinant	Reduction in pathogenicity?	Reference
Detoxification of: (a) Pre-formed inhibitors	<i>Gaeumannomyces graminis</i>	Avenacinase	Yes	Osborn <i>et al.</i> (1994)
	<i>Gloeocercospora sorghi</i>	Cyanide hydratase	No	Wang and VanEtten (1992); VanEtten <i>et al.</i> (1994)
(b) Phytoalexins	<i>Nectria haematococca</i>	Pisatin demethylase	No	Wasmann and VanEtten (1996)
		Maackiain detoxification (MAK1 gene)	Yes	Enkerli <i>et al.</i> (1998)
Toxin production: (a) Non-specific	<i>Gibberella pulicaris</i>	Trichodiene synthase	Yes	Desjardins <i>et al.</i> (1992)
(b) Host-specific	<i>Cochliobolus carbonum</i>	HC-toxin synthetase 1	Yes	Panaccione <i>et al.</i> (1992)
	<i>Alternaria alternata</i>	AAL-Toxin	Yes	Akamatsu <i>et al.</i> (1997)
Overcoming physical barriers	<i>Nectria haematococca</i>	Cutinase	No	Stahl and Schafer (1992)
	<i>Magnaporthe grisea</i>	Cutinase	No	Sweigard <i>et al.</i> (1992)
		Endo-xylanases	No	Wu <i>et al.</i> (1997)
	<i>Cochliobolus carbonum</i>	Endo-xylanases	No	Apel-Birkhold and Walton (1996)
		Endo-PG	No	Scott-Craig <i>et al.</i> (1990)
		Exo- β -1,3 glucanase	No	Schaeffer <i>et al.</i> (1994)
		β -1,3-1,4-glucanase	No	Gorlach <i>et al.</i> 1998
		Trypsin-like proteases	No	Murphy and Walton (1996)
		ExoPG and ExoPL	No	Kelemu and Collmer (1993)
	<i>Pseudomonas viridiflava</i>	PL	Yes	Liao <i>et al.</i> (1988)
	<i>Agrobacterium tumefaciens</i>	PG	Yes	Rodriguez-Palenzuela <i>et al.</i> (1991)
	<i>Botrytis cinerea</i>	PG	Yes	ten Have <i>et al.</i> (1998)
	<i>Fusarium solani</i>	Cutinase	No	Crowhurst <i>et al.</i> (1997)
	<i>Glomerella cingulata</i>	Pectin lyase	No	Bowen <i>et al.</i> (1995)
	<i>Pseudomonas solanacearum</i>	Cellulase	Yes	Roberts <i>et al.</i> (1988)
	<i>Xanthomonas campestris</i>	Cellulase	Yes	Gough <i>et al.</i> (1988)
Other	<i>Ustilago maydis</i>	Mating type	Yes	Banuet (1992)
		Chitin synthase	Yes	Xoconostlecazares <i>et al.</i> (1997)
	<i>Magnaporthe grisea</i>	MAP kinase (PMK1)	Yes	Xu and Hamer (1996)
		cAMP-dependent protein kinase (CPKA)	Yes	Mitchell and Dean (1995); Xu <i>et al.</i> (1997)
		MPG1 (a hydrophobin)	Yes	Talbot <i>et al.</i> (1993)

Table 1.1. Potential pathogenicity genes tested by gene disruption. Adapted from Van Etten *et al.* (1994) and Oliver and Osborn (1995) .

Some examples of potential pathogenicity genes that have been cloned and disrupted are shown in Table 1.1. The effect of gene disruption on pathogenicity

is also shown and it is clear that rarely has pathogenicity been affected. In many cases, in particular cell wall degrading enzymes, there is residual activity, for example the xylanases of *C.carbonum* and *M.grisea* (Apel-Birkhold and Walton, 1996; Wu *et al.*, 1997). This may be due to the presence of multiple or latent genes encoding isoforms of the same enzyme which can compensate for the loss of the target gene. Even where no residual activity apparently remains there may be sufficient activity remaining, not detected by assay, to achieve the function *in planta*. Similarly there may be a different set of genes encoding the same activity which are only induced *in planta*, as was demonstrated for the pectolytic enzymes of *Erwinia chrysanthemi* (Kelemu and Collmer, 1993). In some cases however, unaltered pathogenicity is not accompanied by residual activity and it is usually concluded that the trait is not essential for pathogenicity. This may be true, or it may be that subtle differences in virulence have not been detected. Controlled inoculations in the glasshouse or growth chamber will not detect any difference in fitness of the mutant isolate in the field where it will have to compete with other microorganisms, withstand environmental extremes and may not be present at such high inoculum densities. Equally, the assessment of lesion size on a leaf will not highlight any defects in the mutant at other stages of the life cycle such as sporulation (Oliver and Osbourn, 1995).

VanEtten *et al.*(1994) proposed another interpretation, that some of these genes represent examples of the evolution of functional redundancy for pathogenicity factors. This theory suggests that pathogens have evolved several ways to tolerate a toxic chemical or to prevent its production, or may use both mechanical and enzymic means to penetrate plant tissues. When only one of these mechanisms is disrupted the fungus can compensate; only when several or all redundant mechanisms are lost will there be a significant reduction in pathogenicity.

Some specific examples of potential pathogenicity factors are considered in more detail below.

1.2.1 Detoxification of pre-formed antifungal molecules

Oat roots contain the toxic pre-formed saponin, avenacin. Isolates of the soil fungus *Gaeumannomyces graminis* var. *avenae* are able to infect oat because they produce the enzyme avenacinase, which de-toxifies avenacin (Crombie *et al.*, 1986). Isolates of var. *tritici* which lack the enzyme are non-pathogenic to oat. Targeted gene disruption of the avenacinase encoding gene rendered mutants non-pathogenic on oats but they remained pathogenic on the non-saponin containing host, wheat. This demonstrated that saponin detoxification determines the host range of this fungus (Osbourn *et al.*, 1994). In contrast, gene disruption of the cyanide hydratase of *Gloeocercospora sorghi* failed to prevent this fungus from infecting cyanogenic plants (Van Etten *et al.*, 1994). This contradicted previous circumstantial evidence that conversion of cyanide to formamide by this enzyme enabled fungi that produced it to be pathogenic on cyanogenic plants.

1.2.2 Detoxification of phytoalexins

Phytoalexins are small antimicrobial molecules made by the plant in response to microbial attack and are thought to play a role in plant resistance. Pea plants produce a phytoalexin known as pisatin and the pea pathogen *Nectria haematococca* produces a pisatin demethylase (PDA) which detoxifies pisatin (Van Etten *et al.*, 1989). All pathogenic isolates produce PDA suggesting it was required for pathogenicity to pea. However gene disruption of PDA resulted in isolates still pathogenic to pea (Van Etten, 1994).

1.2.3 Toxin production

Toxins may assist fungal penetration into the host and facilitate colonisation of host tissues, perhaps as a result of suppression of host defences. They have often been postulated to be important in diseases caused by necrotrophic fungi.

1.2.3.1 Non specific toxins

Many fungi produce non-specific toxins that damage many plant species. The trichothecenes are sesquiterpene epoxides produced by a number of *Fusarium* species. The gene encoding trichodiene synthase (*tox5*), involved in toxin biosynthesis, was disrupted in *Gibberella pulicaris*. Mutants showed reduced pathogenicity to parsnip but not potato, suggesting that this toxin may only be important in certain plant interactions (Desjardins *et al.*, 1992).

1.2.3.2 Host selective toxins

The best characterised host selective toxins are found in the genus *Cochliobolus* (reviewed by Walton, 1996). Race T isolates of *C.heterostrophus* are extremely virulent on maize containing Texas type cytoplasmic male sterility (Tcms). This virulence is attributed to the production of a T-toxin. Race O of *C.heterostrophus* does not produce T-toxin and is a minor pathogen of maize regardless of cytoplasm. Generation of Tox⁻ mutants in race T results in a reduction in the ability to cause disease. *C.carbonum* race1 is pathogenic to certain maize varieties which lack the ability to detoxify the HC-toxin produced by the fungus. These varieties are resistant to non-toxin producing isolates and gene disruption mutants which are Tox⁻ are non pathogenic.

Also well studied are the host-specific toxins of *Alternaria* pathogens. There are now nine or more *Alternaria* pathogens that produce host-specific toxins, including three pathotypes of *A.alternata*. The AAL-toxin of *A.alternata* affects tomato and its disruption makes the mutant non-pathogenic (Akamatsu *et al.*, 1997).

1.2.4 Cell wall degrading enzymes (CWDE)

While *S.nodorum* is known to produce phytotoxins in culture their production *in vivo* has not been demonstrated. It produces two toxins, septorine and

mycophenollic acid (Keller *et al.*, 1994). Septorine has a decoupling action on mitochondria isolated from wheat coleoptiles while mycophenollic acid acts by directly inhibiting the Calvin cycle. No correlation between toxin production and pathogenicity has been established, although the toxins may produce necrotic symptoms which occur in terminal stages of the disease in the field (Kent and Stobel, 1976).

The ability of *S.nodorum* to penetrate the host directly or from unmelanised appressoria, combined with the observed diffuse wall around the penetration site and also the subsequent largely intercellular growth of *S.nodorum* implicate a possible role for cell wall degrading enzymes in host penetration and colonisation. CWDE will therefore be treated in more depth than the previously described potential pathogenicity factors. The composition of the plant cell wall will be described alongside the nature of the enzymes required to degrade it. Finally, the involvement of these CWDE in plant pathogenesis will be discussed.

1.3 The Plant Cell Wall

The plant cell wall is a complex heterogeneous structure. The exact composition and structure of the wall varies depending on plant genus, age, environment and location within the plant. The structure and function of the wall will be examined, with particular reference to differences in dicots and graminaceous monocots and the types of linkages in the wall. Information has been compiled from Carpita and Gibeaut (1993), Showalter (1993) and Brett and Waldron (1996). This is with the view to understanding the nature of the wall that pathogens encounter and need to break down.

The enzymes required for wall degradation will also be considered along side their substrates. Cell wall degradation occurs in a number of situations including, developmental stages in the plant such as abscission, during pathogen attack or during digestion by herbivores.

1.3.1 Function of the wall

The plant cell wall serves many functions. The rigidity of the wall provides mechanical strength and shape to the cell but still allows the cells to expand by reversible weakening of parts of the wall. The cross-linked macromolecules that form the wall may control transport of large molecules in and out of the cell and the walls of neighbouring cells in direct contact with one and another form the apoplast, a major transport pathway. The impermeability of the wall also acts as a barrier to potentially pathogenic organisms, and further modifications such as lignin or callose deposition can enhance this capability. Oligosaccharides derived from the wall may play a role in cell signalling during pathogen and insect attack, mechanical damage, cell elongation and cell differentiation. Finally, the wall plays a role in the storage of food reserves and its metabolic turnover is under tight control. This increases dramatically at certain points in development such as seed germination, fruit-ripening, abscission and senescence. The range of functions that the plant cell wall serves explains the complexity of the wall structure and the necessity for its biosynthesis to be under close control.

1.3.2 Structure, composition and enzymic degradation of wall polymers

The wall is typically composed of three layers. The first layer, the middle lamella is formed from the cell plate laid down at cell division between the two daughter cells. When this thin layer is complete the daughter cells begin to deposit the next layer, the primary cell wall. This layer will continue to be deposited as long as the cell is still expanding, maintaining its thickness of 0.1-1.0 μm . After this expansion ceases, a secondary wall may be deposited inside the primary wall in some tissues. Many cells only have the first two layers, for example epidermal cells (Smart, 1991). Where the secondary wall is deposited it can be morphologically and chemically variable and is usually thicker than the primary wall.

All three wall layers are composed of two domains: a microfibrillar domain and a matrix domain. The microfibrillar domain is composed of cellulose and can be distinguished from the matrix domain by its relative homogeneity and its high degree of crystallinity. The matrix domain is heterogeneous in contrast, and can be composed of pectins, hemicelluloses, proteins, and phenolics.

1.3.2.1 The microfibrillar domain

This phase is so termed because it comprises extremely long, thin structures called microfibrils, typically 10nm in width and spaced 20-40nm apart. Each microfibril is made up of cellulose molecules, aligned parallel to the long axis of the microfibril with about 30-100 molecules lying side by side at any one point along the microfibril. Cellulose is an unbranched β 1,4-glucan with a chain length of 2-14,000 residues per molecule. These chains are held in a crystalline or paracrystalline lattice within the microfibril, stabilised by both intramolecular and intermolecular bonds, giving a structure of considerable tensile strength. The unsubstituted nature of the long chains allows close association between cellulose molecules and for H-bonds to form. Whilst cellulose is predominantly D-glucose, other sugars have been found associated with it, most frequently, mannose and xylose. These are thought to be part of the paracrystalline region, although their exact association with cellulose has yet to be determined.

Cellulose may be absent from the middle lamella while in the primary wall it can constitute 20-30% of the wall and 40% of the secondary wall. In the primary wall fibrils are more randomly distributed compared to secondary walls. In some cell types such high levels of cellulose are not observed. In certain tissues, such as the endosperm of some monocots, particularly grasses, cell walls contain only 5% cellulose (Smart, 1991). These cellulose microfibrils provide a framework for the matrix polysaccharides.

The insoluble, partly crystalline, microfibrillar structure of cellulose makes it very resistant to enzymic attack and only a small minority of bacteria and fungi

are capable of degrading native cellulose (Goyal *et al.*, 1991). Cellulose degradation therefore requires the synergistic action of at least three classes of hydrolytic enzymes: exo-1,4- β -cellobiohydrolase, endo-1,4- β -glucanase and β -glucosidase (Ward and Young, 1989). Cellobiohydrolase has the highest affinity for cellulose and attacks the non-reducing end of the cellulose chain, releasing primarily cellobiose, the glucose dimer. Exo-1,4- β -cellobiohydrolases may also possess some endo-type activity and may release cellotetraose first before catalysing further breakdown into cellobiose and small amounts of glucose (Okada and Tanaka, 1988). Endo β -1,4 glucanases cleave internal β -1,4 glucosidic linkages and hence decrease the polymerization of long chain β -1,4 glucose polymers but in general do not attack crystalline cellulose. Instead they act on amorphous regions in the cellulose fibres, opening up new chain ends for attack by cellobiohydrolase. β -Glucosidase (or cellobiase) attacks the β 1,4 glucosidic linkage in cellobiose and other short oligosaccharides to release glucose. This enhances the degradation process by removing cellobiose, the end product repressor of cellobiohydrolase and endoglucanase (Enari and Niku-Paavola, 1987).

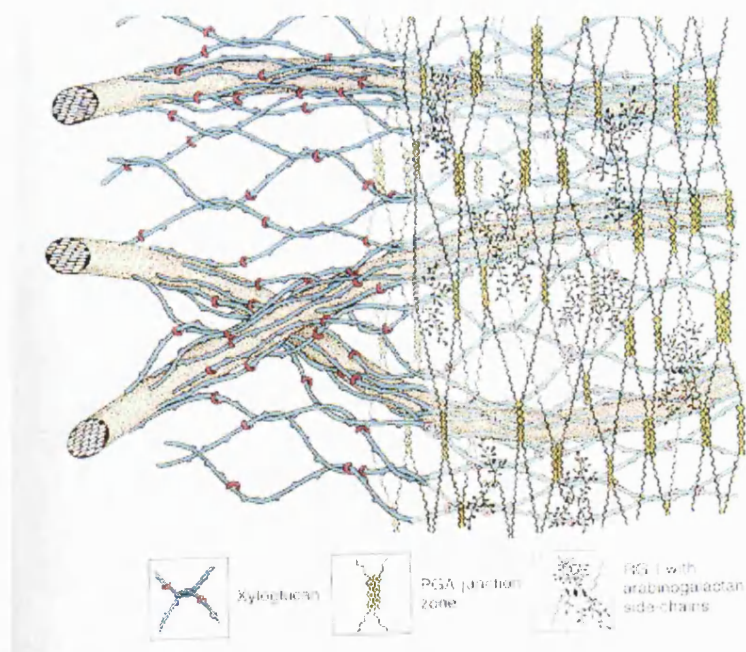
1.3.2.2. The matrix domain

The matrix domain is an extremely heterogeneous phase, consisting of polysaccharides, proteins and phenolic compounds. The structure and composition of the wall with respect to each polymer varies in different parts of the wall, different types of cell, in different species and under different environmental conditions (Table 1.2)

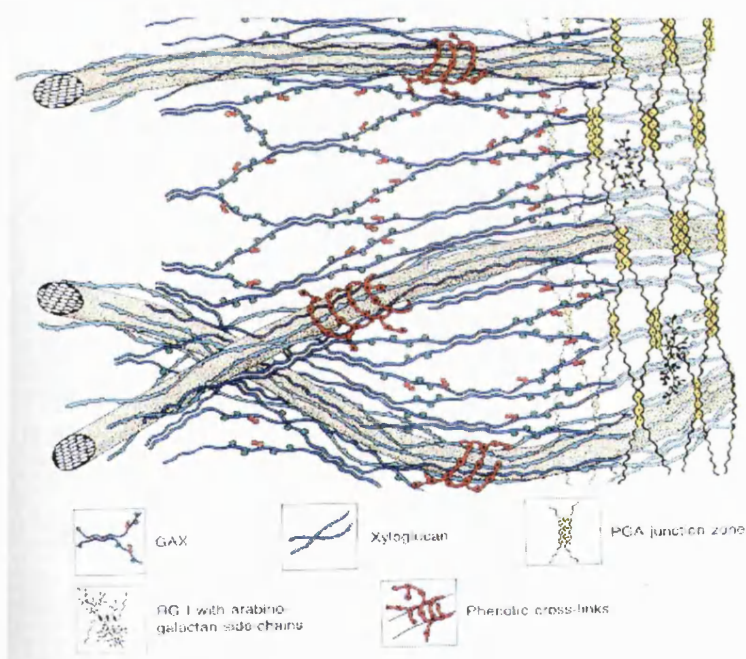
Component	Location	% Dry Wt primary cell wall (dicot)	% Dry Wt primary cell wall (graminaceous monocot)
Pectin -rhamnogalacturonan I -arabinan -galactan -arabinogalactan I -homogalacturonan -rhamnogalacturonan II	Middle lamella and primary cell wall	35%	8-9%
Hemicelluloses -xylan -glucomannan -galactomannan -glucuronomannan -xyloglucan -callose (β 1,3-glucan) - β 1,3-, β 1,4-glucan -arabinogalactan II	Mainly primary cell wall, but also middle lamella and secondary cell wall	15-25%	40%
Proteins -extensin -arabinogalactan-protein -glycine-rich proteins -enzymes -lectins	Throughout	10%	10%
Phenolics -lignin -ferulic acid -others e.g. coumaric acid	Throughout after the secondary wall has been deposited	5-10%	5-15%
Water	Throughout	80% of fresh weight	80% of fresh weight

Table 1.2. Showing the main plant cell wall components and their distribution in dicotyledon and graminaceous monocotyledon primary cell walls. Compiled from a number of sources, Brett and Waldron (1990); Fry (1988) and McNeil *et al.*(1984).

Because of the considerable differences in wall structure between dicotyledons and graminaceous monocot plants, two generalised plant cell wall structures have been proposed (Fig 1.5 (Carpita and Gibeaut, 1993)). Type I cell walls are found in dicotyledons and most non-graminaceous monocots while type II cell walls represent the Poaceae eg. graminaceous monocots. While the microfibrillar domain is similar in both types of wall marked differences exist between the matrix components. Type I primary cell walls are typically pectin-rich and the main hemicellulose is xyloglucan.



(A)



(B)

Fig.1.5. (A) Type 1 primary cell walls of most flowering plants. (B) Type II cell walls of the Poaceae (Reproduced from Carpita and Gibeaut, 1993).

1.3.2.2.1 Pectic polysaccharides

There are two main pectic polysaccharides: polygalacturonic acid and rhamnogalacturonan I (RGI). Polygalacturonic acid can be highly methylesterified and in this state is known as pectin. RGI has side chains of arabinose and galactose and may be covalently attached to polygalacturonic acid.

These pectic polysaccharides are degraded by hydrolytic polygalacturonases (PG) or by lyases (PL) which result in an unsaturated bond between carbons 4 and 5 at the non-reducing end of the broken chain (Cooper, 1984). Both these enzymes may attack the RGI chain internally at random (endo-) or terminally (exo-) and some even combine both (Cooper *et al.*, 1978). Endo-enzymes are the most destructive; cleavage of 1% of the glycosidic bonds can cause a 50% decrease in substrate viscosity. In contrast, exo-enzymes must cause 20-40% hydrolysis to effect a similar change (Cooper, 1983). Enzyme specificity may also be observed for regions either with methylated or free carboxyls. These methoxy groups can be removed by pectin methylesterase allowing other depolymerases specific to free carboxyls to act. PGs usually have acidic pH optima (4-5) whereas lyases are most active at high pH (8-10) and have a requirement for Ca^{2+} (Cooper *et al.*, 1978).

1.3.2.2.2 Xyloglucan

Xyloglucan is the principal hemicellulose in the primary walls of dicots and consists of a backbone of β 1,4-linked glucose residues, to the majority of which xylose residues are attached by α (1-6) bonds. Xyloglucan can be degraded by endo β -1,4-glucanases, producing small fragments that can no longer bind to cellulose (Bauer *et al.*, 1973).

Type II cell walls in contrast to type I are low in pectic polymers and xyloglucan and are instead abundant in glucuronoarabinoxylan and glucans (Fig.1.5).

Because the wheat cell wall has a type II structure these components will be considered in more detail than those of the type I walls.

1.3.2.2.3 Glucuronoarabinoxylan (GAX)

This is the main interlocking polymer in the type II cell wall, largely replacing xyloglucan and pectin. Type I primary walls contain only small amounts of a glucuronoarabinoxylan while secondary walls contain glucuronoxylan as the major hemicellulose. Xylan consists of a backbone of β 1,4-linked xylose residues with various substitutions on some xylose residues; α -linked 4-O-methylglucuronic acid on C2, α -linked arabinose on C2 or C3 and acetyl esters on C2 or C3 (up to 50%) Fig.1.6.

Substituents will vary with cell type and occasionally longer side chains of arabinose can be found. In type II walls GAX predominates in the primary wall while the secondary wall contains an arabinoxylan with rather more glucuronic acid. The degree of branching greatly affects their ability to bind to each other; the unbranched β 1-4 xylans can H-bond to cellulose or to each other. Substitutions prevent the formation of H-bonds and cause the xylan to be water soluble. Hence in dividing elongating cells highly branched glucuronoarabinoxylans are abundant, whereas after elongation more unbranched GAX accumulates.

GAXs are cross-linked in walls by both esterified and etherified hydroxycinnamates and other phenolic substances. Ferulate and *p*-coumarate esters form by attachment to the arabinose units of GAX (Fig.1.7). Diferulate esters may then form between neighbouring feruloyated GAX chains and can cross-link a portion of the GAX matrix. While unbranched GAX may bind to cellulose the branched GAX functionally replace pectins in the wall, with the

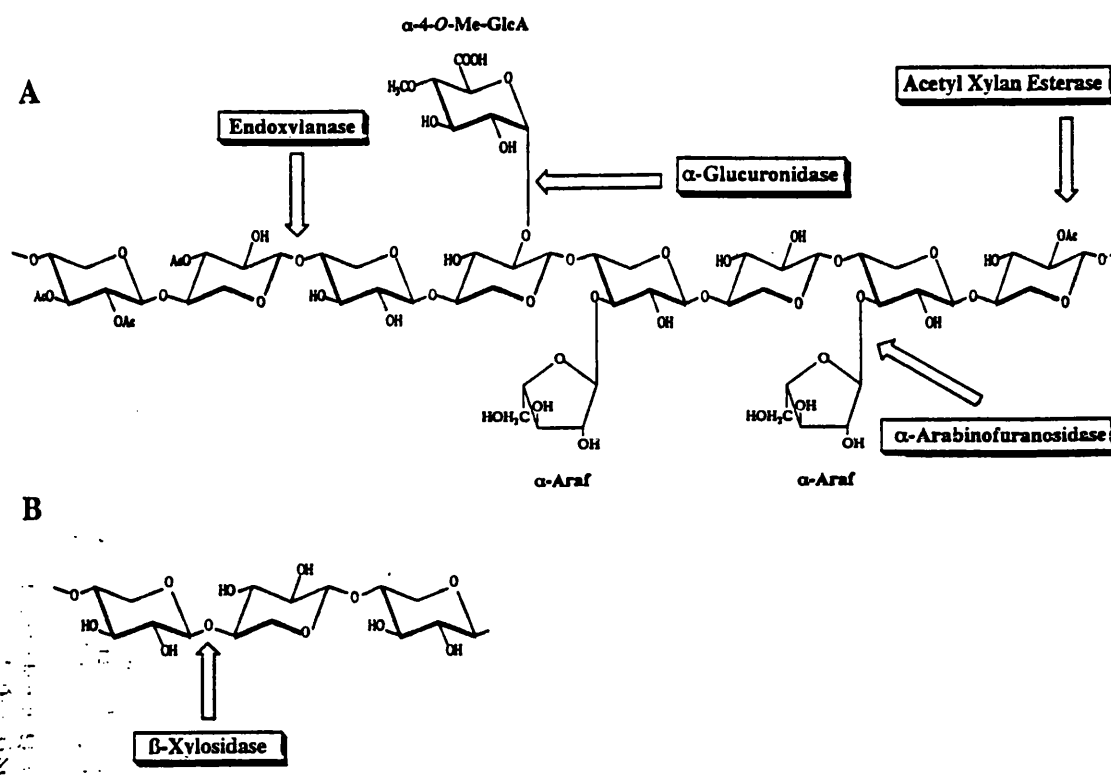


Fig.1.6. (A) The structure of xylan and the xylanolytic enzymes involved in its degradation. Ac: Acetyl group; α -Araf: α -arabinosidase; α -4-*O*-methylglucuronic acid. (B) Hydrolysis of xylooligosaccharide by β -xylosidase. (Reproduced from Sunna and Antranikian, 1997).

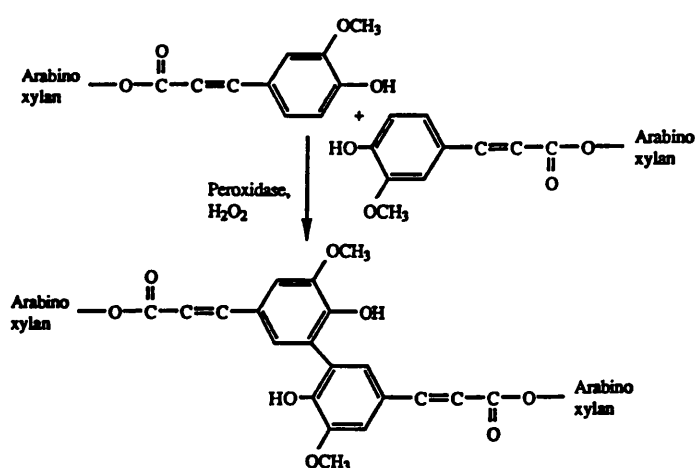


Fig.1.7. Formation of diferulic acid in grasses (Reproduced from Jeffries, 1990).

spacing of arabinose and glucuronic acid substitutions determining porosity and surface charge.

Because of the heterogeneity of glucuronoarabinoxylan, its hydrolysis requires the synergistic action of a number of endo- and exo- acting enzymes (Fig.1.6). While some exo- β 1,4-xylanases exist the majority are endo- β 1,4-xylanases which cleave internal glycosidic linkages in the main xylan backbone. These are described in detail in Chapter 5. In contrast to pectin lyase and polygalacturonase the attack on the substrate is not random; hydrolysed bonds depend on the nature of the substrate e.g. length and degree of branching or the presence of substituents (Wong *et al.*, 1988). Xylooligosaccharides are initially released following xylanase action and these will be further hydrolysed to xylotriose, xylobiose and xylose. Endo-xylanases can be classified on the end products released from the hydrolysis of xylan e.g. xylose, xylobiose and xylotriose, and/or arabinose. Thus, xylanases can be classified as nondebranching (arabinose nonliberating) or debranching (arabinose-liberating) enzymes (Dekker and Richards, 1976). The majority of endo- β 1,4 xylanases are nondebranching, but a number of arabinose-liberating xylanases have been characterised (Wood and McCrae, 1986a). Many organisms are capable of producing both types of xylanase, resulting in the maximum efficiency of xylan hydrolysis (Wong *et al.*, 1988). Xylan degradation is also assisted by removal of side groups by accessory enzymes such as α -L-arabinofuranosidase, acetylsterase and α -glucuronidase. Xylobiose is hydrolysed to xylose by β -D-xylosidase, an exo-glycosidase which acts on short xylooligosaccharides (Sunna and Antranikian, 1997). In addition to these glycosidases, ferulic acid and *p*-coumaric esterases may be important in cleaving the diester linkages between GAX chains (Jeffries, 1990).

1.3.2.2.4 β 1,3-, β 1,4-glucans

These mixed-link glucans are important wall components in cereals and other grasses. They are unbranched and the ratio of 1,3 to 1,4 links is between 1:2 and 1:3 and usually 1,3-linked residues separate sequences of two to four 1,4-linked

residues. In barley, mixed-linked glucan is covalently bound to wall protein and this may be true in other cereals. They may also hydrogen-bond with other β 1,4 linked polysaccharides. Another hemicellulose, callose, is a β 1,3-glucan and is able to form gels under certain circumstances and can accumulate adjacent to the wall in response to wounding.

Mixed linked glucans are degraded by β 1,3-1,4-glucanases, known as mixed linked glucanases. Hydrolysis of the mixed 1,3/1,4- β -D-glucans appears to involve cleavage of 1,4-linkages adjacent to 1,3 bonds (Ward and Young, 1989). These enzymes can also degrade other glucans e.g. β 1,3-glucans and β 1,4-glucans. Specific β 1,3 or β 1,4 glucanases cannot degrade the mixed linked glucans and specific β 1,3 glucanases are thought to be important in callose degradation (Van Hoof *et al.*, 1991).

1.3.2.2.5 Proteins

The protein content of cell walls varies with cell type and environment, but is generally less than 10% and is important in both type I and type II walls. Most wall proteins are glycosylated and the most abundant ones contain an unusual amino acid, hydroxyproline which is not generally found in the proteins of the protoplast. These are known as the hydroxyproline-rich glycoproteins (HRGPs) and the best studied group containing these are the extensins.

About 40-50% of the weight of extensin is protein and the molecular weight of the monomer is around 40,000. In dicots, extensins are particularly abundant and as a result they are the best characterised. Up to 40% of amino acids in extensins from dicots are hydroxyproline, together with a large amount of serine and lysine. Hydroxyproline frequently occurs as part of a sequence of Ser-(Hyp)₄ repeats, separated by an average of six amino acids. The repeating hydroxyproline results in a molecule with a helical secondary structure and appears as a stiff rod in electron micrographs. The hydroxyproline residues are sites of attachment for tri and tetra-arabinose oligosaccharides and the serine residues are attachment points

for single galactose residues. The tri and tetra -arabinose oligosaccharides wrap around the protein rods, reinforcing the structure and their removal results in the loss of rod structure. The molecule also contains tyrosine residues, often present as flexible regions of Tyr-Lys-Tyr repeats, which are able to cross-link in the wall to form intramolecular isodityrosine units and perhaps also intermolecular covalent bridges. Monocots contain glycoproteins which have some homology to dicot extensins, including some which are also rich in threonine (THRGP) and histidine and alanine (HHRGP) (Carpita, 1996). In the graminaceous monocot maize, both THRGP and HHRGP are present and these are distinct from dicot extensin. The THRGP is rich in threonine and proline in addition to hydroxyproline, lysine and serine. It contains two novel amino acid repeat motifs but only one "signature" Ser-(Hyp)₄ sequence near the carboxy terminus, instead the majority of the repeats contain a Lys substitution for a Hyp (Kieliszewski *et al.*, 1990). Also in contrast to dicot extensin the serine residues and approximately half of the hydroxyproline residues are not glycosylated (Lamport and Millar, 1971). THRGP is also predicted to exist in a random coil conformation, not in a polyproline helix. Sorghum and rice have also been shown to contain similar THRGP sequences suggesting they may be present in other graminaceous monocots (Showalter, 1993).

The extensive cross-linking of extensin molecules makes them difficult to extract and study. Hence much of the information is based on gene sequences and salt extractable precursors. The extreme insolubility of extensin has led to the proposal that it has a structural function and its formation is thought to be associated with cessation of growth. Extensin precursors can be seen early in cell wall formation but a large increase in extensin occurs as the cell stops growing. Other proteins may be necessary to lock the extensins together. One such protein may be a 33kDa proline-rich protein or PRP also found in nodulins. These are highly expressed later in cell development and can be induced within a few hours of wounding compared to a full day before the appearance of extensin. One theory is that the precursors of extensin observed in the developing walls can't crosslink without PRPs.

Extensin has also been implicated in resistance of cell walls to fungal infection. Increased extensin deposition and cross linking should lead to a more impenetrable cell wall barrier; levels of extensin have been observed to increase in response to pathogen infection (Showalter *et al.*, 1985).

Another related protein occurs as part of arabinogalactan proteins (AGPs). The polysaccharide portion is similar to arabinogalactan II while the protein portion which comprises 2-10% of the weight is rich in hydroxyproline, serine, alanine and glycine. These two components are probably linked through a β -galactosyl linkage.

Glycine-rich proteins (GRPs) are also present in walls, probably at the wall-plasma membrane interface and comprise Gly-X repeating motifs where often the X is also a Gly which can make up two thirds of the amino acids. They are associated with walls at the onset of lignification and may act as nucleation sites for lignin formation.

Many enzymes are also associated with the wall and include peroxidases, invertases, proteases, cellulases, acid phosphatase, pectinases, pectin methylesterase and malate dehydrogenase. A number of exoglucosidases have been reported, including β -glucosidase, β -xylosidase, α -galactosidase and β -galactosidase. Endo β 1,4-glucanase and β 1,3 glucanases are also present; the former may be involved in cell wall turnover, especially of xyloglucan. Another enzyme, xyloglucan endotransglycosylase (XET), may be involved in insertion of xyloglucan into the wall and possibly in control of wall extensibility. Also involved in cell wall extension are a class of wall proteins called expansins (McQueen-Mason *et al.*, 1992). Finally, the wall also contains lectins (proteins that bind specifically to certain sugars without acting enzymically on them) such as arabinogalactan-proteins which bind to β -linked sugars, for example the β -linked galactose residues in pectin side-chains.

Characterisation of enzymes involved in the degradation of cell wall proteins has not been as comprehensive as that for the polysaccharide degrading enzymes described previously. Because of the extensive glycosylation of proteins such as extensins they are innately resistant to degradation by proteases (Lampert, 1980). Cell wall protein proteins often require prior deglycosylation by acid hydrolysis, treatment with hydrogen fluoride or enzymatic treatment with arabinosidase before they are susceptible to proteolytic degradation. Until recently very few proteases had been demonstrated to be capable of degrading a plant cell wall protein without prior deglycosylation (Heilbronn *et al.*, 1995; Willis *et al.*, 1987). However, a metalloprotease has been isolated from *Xanthomonas campestris* that specifically degrades proline/hydroxyproline rich wall glycoproteins (Dow *et al.*, 1998).

1.3.2.2.6 Lignin and other phenolic compounds

The phenolic polymer lignin is found in certain differentiated cell types such as sclerenchyma and xylem vessels and tracheids, after cell elongation has ceased and in certain tissues in response to infection. The precursors coumaryl, coniferyl and sinapyl alcohols are linked irregularly following the non-enzymic polymerisation process. This molecule continues to polymerise and fill any spaces in the wall as long as space is available. As this continues water is displaced from the wall and a strong hydrophobic meshwork surrounding the other wall components is formed. This holds the wall in place and growth ceases. These fully lignified cells are dead and provide protection for other cells against pathogen infection.

Other phenolic compounds may be present in the wall, the most important of which is ferulic acid. In dicots, ferulic acid may be involved in cross-linking pectins through esterification to arabinose and galactose residues in pectin. In cereals which only have low amounts of pectin, ferulic acid is linked to the arabinose of arabinoxylans. Also present in cereal walls is *p*-coumaric acid (Jeffries, 1990).

1.3.2.3 Linkages between different wall polymers

1.3.2.3.1 Covalent linkages.

There is good evidence for covalent cross-linking of pectin *via* diferulic acid. Ferulic acid is esterified to arabinose and galactose, and two ferulic acid units can be linked by peroxidase activity to form a diphenyl bond. Glucuronoarabinoxylan is also cross-linked in a similar way. Another peroxidase-catalysed bond may occur between tyrosine residues of extensin. This results in the phenolic ether linkage of isodityrosine, known to occur intramolecularly within extensin and may also occur intermolecularly. Tyr-Lys cross-links have also been suggested as possible intermolecular bonds. Extensin is synthesised and secreted through the plasma membrane as a water-soluble precursor protein. Once in the wall it is linked into an insoluble extensin network. The elongated extensin molecules lie perpendicularly to the cellulose microfibrils and their associated hemicelluloses and are thought to hold the layers together.

1.3.2.3.2 Non-covalent linkages

The polyhydroxylic nature of the main wall polymers makes it likely that a large number of hydrogen bonds will form within the wall. These become structurally significant when a substantial number form between two macromolecules. One of the most important of these types of interactions will be the anchoring of cellulose microfibrils (held together by hydrogen bonds) to the matrix hemicelluloses. In turn many of these matrix hemicelluloses are held together by hydrogen bonds, for example pairs of xyloglucan molecules. Ionic bonds will occur between polymers that contain charged groups, such as extensin, with a net positive charge, and the negatively charged galacturonans. Ionic binding also occurs between two stretches of contiguous galacturonic acid residues, with calcium ions acting as ionic bridges between the negatively charged galacturonate

residues. Around 15-20 contiguous unsubstituted galacturonic acid residues are needed for stable complexes to be formed and these 'egg-box' linkages occur frequently enough to give rise to a gel-like structure in the wall. Finally, hydrophobic forces may occur between protein molecules and between proteins and lignin.

Interactions between the many different wall polymers will result in overall wall structure. The linkages that form between them and the physical entanglement of large, irregular cell wall macromolecules will contribute to the strength of the wall. A complete model of the overall structure of the wall is currently impossible because of the extreme complexity of its components and their interactions. Modelling 3D structure is very difficult due to problems with alteration of structure during extraction and study of wall components. Hence, the structures discussed above merely represent the structural skeleton of the wall and not the entire biological structure. Little is currently known about the metabolic machinery of enzymes involved in cross-linking, hydrolysis and turnover of polysaccharides and proteins and in the formation of lignin. It is clear however that the plant cell wall is a complex, dynamic structure and that pathogens attempting to penetrate it will require a range of depolymerase enzymes to be successful.

1.4 Cell Wall Degrading Enzymes in Plant Pathogenesis

CWDE are produced by the vast majority of pathogenic and saprophytic micro-organisms, including bacteria, nematodes and fungi (Walton, 1994). Therefore the presence of CWDE alone is not evidence for their involvement in pathogenicity. However the rapid and extensive degradation of host cell walls observed during infection by some necrotrophic pathogens e.g. *Botrytis cinerea* (Mansfield and Richardson, 1981) indicates a role for cell wall degrading enzymes in pathogenicity. Similarly, the more localised degradation of cell walls observed in biotrophic interactions e.g. *Uromyces* spp. where host cells are not

killed shows the level of cell wall degradation is adapted to the nutritional mode of the fungus (Cooper, 1981).

Pathogens may require CWDE for penetration of the leaf surface and subsequent inter/intracellular growth through the plant. CWDE may also provide a source of nutrients obtained from the wall polymers; plant cell walls are the world's most abundant source of glucose and other sugars (Walton, 1994). Finally CWDE may be important for free saprophytic growth, such as during overwintering on alternative hosts. Pathogens may require a different set of enzymes to achieve this.

Given the complexity of the plant cell wall a wide range of enzymes are required to degrade the many different polymers that constitute the plant cell wall (Table 1.3). All of these enzymes have been shown to be produced by at least one plant pathogen (Walton, 1994).

Cellulases
Endo- β -1,4-glucanase
Cellobiohydrolase
β -Glucosidase
Pectinases
Pectate lyase (endo- and exo-)
Endo pectin lyase
Polygalacturonase (endo- and exo-)
Pectin methylesterase
β -1,4-Xylanase (endo- and exo-)
β -Xylosidase
α -Arabinofuranosidase
Arabinase
α -Glucosidase
β -1,3-Glucanase (laminarinase)
β -1,3- β -1,4-Glucanase (mixed-linked glucanase)
α -Galactosidase
β -Galactosidase
β -1,4-Galactanase
Protease

Table 1.3. Cell-wall-degrading enzymes reported from plant pathogens. Adapted from (Walton, 1994).

Most of these CWDE are secreted extracellular enzymes but some such as β -glucosidase and β -xylosidase may have intracellular as well as extracellular forms, degrading the soluble products of other extracellular enzymes (Walton, 1994). CWDE are usually subject to catabolite repression and substrate induction (Cooper, 1983). However, some are constitutively expressed. In a study by Cooper *et al.* (1988) the xylanase of *R.cerealis* was induced by polymers containing xylose, i.e.xylan and cell walls. In contrast arabanase and laminarinase were produced on a range of polymers and even on glucose showing that these are constitutively expressed and not subject to catabolite repression. Similar constitutive expression of other enzymes by other pathogens has been detected, such as pectinases of *S.sclerotiorum* (Riou *et al.*, 1991) and endoglucanase and xylanase of *C.gloeosporioides* (Ortega, 1994). The constitutive expression of these CWDE presumably shows the long term adaptation of these pathogens to their host cell wall. This is also supported by the co-ordinate regulation of some cell wall degrading enzymes; the pectinase genes of *Erwinia chrysanthemi* are coregulated with other extracellular enzymes (Barras *et al.*, 1994; Condemine and Robert-Baudouy, 1995). In addition some cell wall degrading enzymes may be developmentally regulated. *Uromyces viciae-fabae* produces a range of differentiation specific CWDE during infection structure differentiation on artificial substrata (Deising *et al.*, 1995).

Which of these enzymes are important in causing disease has been hard to assess. Each of the polymers in the wall differ in their accessibility, and may only be attacked once other polymers have been degraded. Equally, some polymers such as cellulose are more resistant to degradation so may be degraded more slowly. Also, degradation of certain polymers, such as pectins which hold the wall together, may be more destructive than degradation of other polymers, such as cellulose. Monomeric sugars of some polymers may be better nutrient sources; for example is xylose can be utilised more efficiently than glucose (Flannigan and Sellars, 1978). As a result of all these factors, different depolymerases may be equally important but at different stages of infection or of the life cycle. The

study of cell wall depolymerases has also been dictated by the commercial availability of substrates for known wall polymers and linkages. Other CWDE may exist that are as yet undiscovered because we don't know their substrates.

The starting point for most studies on CWDE centres on the range of CWDE produced by a pathogen when grown *in vitro* on host cell walls. Insoluble, extracted host cell walls act as highly effective inducers of enzyme synthesis when used as sole carbon source (Cooper, 1977). In a study by Cooper and Wood (1975) on *Verticillium albo-atrum* and *Fusarium oxysporum* CWDE production was sequential in the order of pectic enzymes, hemicellulases, cellulase and seemed to reflect the physico-chemical susceptibilities of the wall polymers. Several other fungal pathogens of dicot hosts respond in a similar way (Cooper, 1983). That pathogens can adapt their CWDE depending on the nature of the host cell wall was demonstrated by Cooper *et al.* (1988). Three cereal pathogens, including *Rhizoctonia cerealis* were grown *in vitro* on wheat cell walls. All three produced a similar sequence of CWDE, principally arabanase, xylanase and laminarinase (β -1,3 glucanase). In contrast to pathogens of dicotyledons only low levels of pectic enzymes were produced, reflecting the differences in primary cell wall structure. Interestingly, when *R. cerealis* was grown on potato stem walls instead of wheat cell walls the main enzymes observed were β -galactosidase and pectin lyase. Correspondingly only low levels of xylanase were produced, again reflecting the composition of the host cell walls. Observing CWDE production *in vitro* alone is insufficient evidence for their involvement in pathogenicity and a number of criteria for assessing the role of an enzyme in pathogenicity were suggested by Cooper (1984). These include detection *in vivo*; ultrastructural changes and enzyme localisation, degradation of insoluble host walls and reduction of pathogenicity by enzyme inhibitors.

Further evidence for activity of CWDE *in planta* is the observation that oligosaccharides released by their action or the enzymes themselves can elicit defence responses in the host. These include responses such as production of pathogenesis related proteins, ethylene, proteinase inhibitors, lignin, activated

oxygen, phytoalexins, and inhibition of protein synthesis, stimulation of membrane K^+/H^+ exchange and necrosis (Walton, 1994). This evidence suggests that if wall-degrading enzymes are required by pathogens then detection of released oligosaccharides would be advantageous to the host plant in mounting a defence response. PG and PL elicit responses by the production of elicitor-active oligogalacturonides, the best studied of the oligosaccharides (Ryan, 1988). Also, plants contain proteins, called PG-inhibitor proteins (PGIPs), that modulate the activity of endoPG and thereby increase its elicitor activity (Cervone *et al.*, 1989). Oligogalacturonides with a degree of polymerisation between 10 and 15 are most active. Several purified fungal endo- β -1,4-xylanases also induce an array of host defence responses and their small size enables them to be transported in the xylem. Their elicitor activity is due to xylanases acting directly rather than as a result of the products of their action on the wall (Sharon *et al.*, 1993). Sensitivity to xylanase is controlled by a single dominant gene in tobacco (Bailey *et al.*, 1993). This raises the possibility that this ability to detect the fungal CWDE may play a role in specific disease resistance, which is often under monogenic control. This theory however requires further investigation.

A large amount of circumstantial evidence implicating CWDE in disease has been obtained and more recently this has been tested by the use of gene disruption. This has enabled the effect on pathogenicity of the removal of a single CWDE to be studied. However these studies have not been as definitive as they may have first appeared.

1.4.1 Cutinases

The outer surface of the plant epidermis and the substomatal cavity are covered with a cuticle layer composed of wax and cutin (Koller, 1991). Cutinase and its role in the infection process has been most extensively investigated for the penetration of pea by *F.solani* f.sp.*pisi* (Kollatakudy, 1985). Principally the use of monospecific antibodies against cutinase from *Fusarium* and the chemical serine-esterase inhibitor DIPF were able to prevent infection of pea stems by

F.solani f.sp.*pisi* (Maiti and Kolattukudy, 1979). The insertion of the gene encoding this cutinase into the wound pathogen of papaya fruits, a *Mycosphaerella* sp, enabled this fungus to infect the host through the intact cuticle (Dickman *et al.*, 1989). Similarly, this new ability to penetrate the cuticle could be inhibited by antibodies against *Fusarium* cutinase. U.V induced mutants of *F.solani* with an 80-90% reduction in cutinase activity showed a 55% reduction in virulence that could be restored by the addition of purified cutinase (Dantzig *et al.*, 1986). This strong biochemical evidence for the role of cutinase in penetration was contradicted by Stahl and Schafer (1992) who created a cutinase-deficient mutant of *F.solani* f.sp.*pisi* by transformation-mediated gene disruption. The mutant showed no reduction in virulence and pathogenicity and it was concluded that cutinase was not required for pathogenicity on pea. In addition no residual cutinase activity or other cutinase activities *in vivo* were detected. However, a subsequent, more detailed analysis of disrupted cutinase mutants has shown some reduction in disease (Rogers *et al.*, 1994). This latter study investigated multiple spore levels and used microscopic examination of developing lesions. It appears therefore that cutinase does play a role in infection in this system.

Alternaria alternata mutants deficient in cutinase showed greatly reduced pathogenicity towards pear and cutinase inhibitors similarly prevent penetration (Tanabe *et al.*, 1988a,b). In contrast, disruption of the cutinase gene of *Magnaporthe grisea* failed to alter pathogenicity; however residual activity remained and there is strong evidence for mechanical penetration (Sweigard *et al.*, 1992). The mucilaginous matrix which surrounds the spores of *C.graminicola* in acervuli contains four cutinases which remain associated with spores following dispersal (Pascholati *et al.*, 1993). Importantly, the addition of the cutinase inhibitor DIPF did not alter appressorium morphogenesis but did prevent the development of disease. This is interesting because *Colletotrichum* spp. form melanized appressoria, similar to those of *M.grisea*, where cutinase is not thought to be required for penetration (Sweigard *et al.*, 1992). The observed inhibition of disease may be caused by problems with adhesion or surface

recognition as well as penetration. Cutinase deficient mutants of *C.gloeosporioides* generated by chemical mutagens or U.V were unable to infect the host, papaya. Infection was restored either by wounding of the papaya fruit or treatment with purified cutinase, suggesting the cutinase is required for penetration (Dickman and Patil, 1986).

Cutinase may also play a role in adhesion. Autoclaved uredospores of *U.viciae-fabae* and spores in the presence of the cutinase inhibitor DIPF fail to adhere to the leaf (Deising *et al.*, 1992). Adhesion of autoclaved spores to the leaf could be restored by the addition of cutinase activity isolated from the surface of uredospores.

1.4.2 Pectinases

Endo-pectinases are the best studied of the CWDE because pure preparations of these enzymes are capable of tissue maceration and (indirectly) cell killing, such as that observed in soft rot diseases (Bateman and Basham, 1976). Because of the importance of pectin in type I cell walls they are considered to be important to many pathogens of dicots (Cooper, 1983). The ability of endopolygalacturonase and pectin lyase from *Verticillium albo-atrum* to degrade isolated plant cell walls has been demonstrated (Cooper *et al.*, 1978). Wall degradation during infection of apple fruit by *Botrytis cinerea*, a soft rot pathogen, has been shown to coincide with a 50-70% depletion of pectic substances from the host cell wall (Cole and Wood, 1961). This was in contrast to infection by a firm rot pathogen, *Sclerotinia fructigena* that only reduced pectic substances by 15%. A more recent study has localised an endopolygalacturonase from *Cochliobolus sativus* to the barley cell wall using immuno-gold labelling, visualised by electron microscopy (Clay *et al.*, 1997). In the same study a corresponding loss of polygalacturonic acid in the immediate vicinity of the penetrating pathogen was also detected using antipectin antibodies. Strong circumstantial evidence for pectinases in disease has not always been supported by studies at the genetic level.

Genetic transformation of the non-phytopathogenic bacterium *E.coli* with the genes encoding the pectic enzymes from the phytopathogen *Erwinia chrysanthemi* resulted in some endo-pectate lyase producing transformants capable of macerating and killing plant tissues (Zink and Chatterjee, 1985). However, a large inoculum was required to initiate colonisation of tissues by these transformants, suggesting that secretion of pectic enzymes alone is insufficient to allow plant parasitism. Since then further studies have focused on generating mutations in pectinase encoding genes to assess their importance in pathogenicity.

The best studied pectinase system is of the bacterium *Erwinia chrysanthemi* which, when grown on pectin, expresses four or five PL genes depending on the strain. When all of these genes were mutated, total pectinase levels declined to less than 0.1% of wild type activity, yet *E.chrysanthemi* remained pathogenic and able to grow on pectin as sole carbon source (Ried and Collmer, 1988). The latter ability could be explained by the remaining presence of an exoPG but this wouldn't be sufficient to cause the observed tissue maceration. In strains where this exoPG and exoPL genes were also mutated pathogenicity still remained. This finally led to the discovery that *E.chrysanthemi* makes an entire new set of pectinases only when growing *in planta* or in cultures supplemented with plant extracts (Kelemu and Collmer, 1993). It was clear from this work that microorganisms rarely make just one enzyme to degrade a particular polymer and those observed *in vitro* may not be produced *in planta*. However, *Pseudomonas viridiflava* has only one PL gene and its mutation prevents it from causing soft rotting on pepper (Liao *et al.*, 1988). Similarly, mutation in the single PG-encoding gene of *Agrobacterium tumefaciens* biovar 3 substantially decreases virulence (Rodriguez-Palenzuela *et al.*, 1991). With regard to fungal pathogenesis, U.V or chemical induced mutants of *Verticillium*, *Fusarium*, *Sclerotinia* and *Alternaria* with reduced pectinase activity failed affect pathogenicity (Walton, 1994). Similarly, targeted gene disruption of the single endoPG gene of *C.carbonum* had no effect on pathogenicity (Scott-Craig *et al.*,

1990). However exoPG activity remained and the fungus was still able to grow on pectin. There also remains the possibility that another endoPG exists *in planta*, therefore it is not possible to conclude whether pectin degradation is essential for pathogenicity in this fungus. Disruption of a pectate lyase gene of *N. haematococca* and a pectin lyase gene of *Glomerella cingulata* also did not reduce virulence, but in both cases other genes encoding these enzymes were present (Bowen *et al.*, 1995; Guo *et al.*, 1995). A U.V. generated mutant of *Colletotrichum magna* lacking extracellular PG or PL activity grew endophytically in its host without producing disease symptoms. Interestingly, it still showed PL mRNA expression and with the use of antibodies it was possible to detect PL accumulation in the hyphae (Wattad *et al.*, 1995). It appears therefore that the mutation prevented extracellular secretion of PL and perhaps PG, giving good evidence for the role of these CWDE in pathogenicity. However, it must be remembered that the mutant was generated by U.V. and hence mutations in other genes may be responsible for loss of pathogenicity. Disruption of an endopolygalacturonase encoding gene from *Botrytis cinerea* revealed that this gene is required for full virulence on tomato leaves and fruits (ten Have *et al.*, 1998). Although the mutants were still pathogenic and displayed similar primary infections compared to controls, a significant decrease in secondary infection, i.e. growth of the lesion beyond the inoculation spot, was observed. This is the first report that disruption of a CWDE gene in a plant pathogenic fungus can reduce pathogenicity. This success may be surprising given that *B. cinerea* has been reported to produce up to 13 different endoPG isoforms. Hence it may be of relevance that the form disrupted in this study was one of only two high pI isoforms, was the only isoform constitutively produced and the only form that has been detected in the outer regions of lesions.

1.4.3 Xylanases

In contrast to the dicot wall where pectin is the main matrix polymer, in graminaceous monocots this is largely replaced by xylan. Xylan is the major interlocking polymer and constitutes up to 40% of the wall. Its degradation could

considerably weaken the wall and allow access for cellulases to their substrate. Hence as pectinases may be important in pathogenesis of microorganisms on dicots so endo- β -1,4-xylanase and β -xylosidase may be crucial for pathogens of graminaceous monocots. The circumstantial evidence for xylanases in disease is not as extensive as that for pectinases, particularly because of their inability to cause tissue maceration directly. Despite this, xylanases have been demonstrated to be capable of degrading isolated cell walls (Cooper *et al.*, 1978; Baker *et al.*, 1977) and of causing depletion of xylose in infected tissue (Bateman and Basham, 1976). Xylanase activity was claimed to be responsible for the death of suspension-cultured rice cells (Ishii, 1988) and their ability to elicit host defence responses (Sharon *et al.*, 1993) has already been described. Accessory enzymes, such as α -arabinosidase, also important in arabinoxylan degradation, may play a role in wall degradation during infection. α -Arabinosidase production has been correlated with pathogenicity of induced mutants of *Monilinia fructigena* (Howell, 1975) and activity has been detected in infected fruits, together with the breakdown product arabinose (Byrde *et al.*, 1973).

Gene disruption experiments to date have failed to support a role for xylanases in infection. *C.carbonum* produces three xylanase degrading enzymes and disruption of the major xylanase gene, *XYL1*, reduced total extracellular xylanase activity by about 90% and eliminated two of the three xylanase isoforms (Apel *et al.*, 1993). Once again this failed to alter pathogenicity but also residual activity remained leaving the question of the role of xylanases in pathogenesis unanswered. Apel-Birkhold and Walton (1996) attempted to address the problem of residual activity by isolating another two xylanase genes and producing mutants in all three xylanase genes. Neither of the two new mutations altered the xylanase activity any more than the original mutation in *XYL1* and the triple mutant also remained pathogenic. The residual activity previously observed therefore is not encoded by *XYL2* or *XYL3* and so this third isoform has now been purified and the gene is being cloned for *XYL4*. Perhaps when this gene is also disrupted pathogenicity may be affected. A similar problem arose following gene disruption of the two major xylanases of *M.grisea* (Wu *et al.*, 1997).

Creation of a double mutant revealed four new xylanase activities, apparently induced in the absence of the other two. Once again these mutants remained pathogenic and the role of xylanase in disease was not determined.

1.4.4 Glucanases

Another important hemicellulose in the walls of some monocots, particularly the Poaceae, is mixed-linked glucan (β 1,3-1,4 glucan). Callose, a β -1,3-glucan is deposited against plant cell walls in response to attempted penetration by fungal plant pathogens (Aist, 1976). Hence degradation of both these types of glucans may be beneficial to plant pathogens. Indeed, the constitutive production of β -1,3-glucanase by *Claviceps purpurea* during infection of cereal florets is accompanied by a failure of callose to accumulate (Dickerson *et al.*, 1978).

An exo acting β 1,3-glucanase produced by *C.carbonum* has been purified and the gene disrupted (Schaeffer *et al.*, 1994) . The mutant remained pathogenic but 66% of wild type activity remained, attributable to two other forms of the enzyme. These other forms however were unable to support growth of the fungus on β 1-3 glucan. *C.carbonum* also produces three extracellular mixed-linked glucanases, two of which are encoded by one gene, *MGL1*. Disruption of this gene also failed to alter pathogenicity but this again may be attributable to the residual activity of the third form (Gorlach *et al.*, 1998).

1.4.5 Cellulases

Cellulose is innately resistant to degradation due to its crystalline structure but degradation of this structural polymer would considerably weaken the wall. Its degradation only appears to occur late on in infection, if at all and hence cellulases were thought unlikely to be important in pathogenesis (Cooper, 1984).

However, disruption of *Pseudomonas solanacearum* endo β -1,4 glucanase gene significantly reduced pathogenicity on tomato (Roberts *et al.*, 1988). In contrast

only a minor reduction in pathogenicity was observed after disruption of the endo β -1,4 glucanase of *Xanthomonas campestris* pv *campestris* (Gough *et al.*, 1988). No reduction in pathogenicity occurred following disruption of the *CEL1* gene of *C.carbonum* encoding a cellulase (Sposato *et al.*, 1995), however residual activities of cellobiohydrolase, endoglucanase and β -glucosidase were detectable in cultures grown on maize cell walls or cellulose.

1.4.6 Proteases

Proteases have been extensively studied in pathogens of animals as potential virulence factors because of the proteinaceous intercellular matrix (Hamer and Holden, 1997). They have also been studied in entomopathogenic fungi which require proteases to penetrate the protein-rich outer integument of insects (St.Leger, 1995). Gene disruption of Pr1a, one of the subtilisin-like proteases produced by *Metarhizium anisopliae*, apparently resulted in a partial loss of virulence against *Manduca sexta* (St.Leger, 1995). Also, when additional copies of this gene were inserted into the genome of *M.anisopliae*, such that Pr1 was constitutively overproduced, challenged larvae exhibited a 25% reduction in time of death compared to the wild type fungus (St.Leger *et al.*, 1996).

Relatively few plant pathogens have been studied for proteolytic activity despite the importance of protein in the cell wall and its possible role in defence (Showalter, 1993). The induction of protease inhibitors in plants following infection by plant pathogens might support their possible role in plant pathogenesis (Ryan, 1990).

Protease production has been detected in a number of plant pathogens, including *Botrytis cinerea* (Movahedi and Heale, 1990b). The aspartic protease produced by this pathogen was detected in infected carrot tissue and was demonstrated to be capable of causing cell death in carrot tissue and carrot suspension cells (Movahedi and Heale, 1990a,b), but could not cause tissue maceration. The role of this protease in disease was further supported by the ability of pepstatin, a

specific inhibitor of aspartic proteases, to reduce disease when mixed with spores prior to inoculation.

There have been few reports of the ability of proteases from plant pathogens to degrade plant cell wall hydroxyproline rich glycoproteins (HRGPs). This is largely thought to be due to the extensive glycosylation of these proteins that makes them innately resistant to proteolytic degradation (Lamport, 1980). A metallo protease produced by *Erwinia carotovora* spp. *carotovora* was able to slowly degrade *in vitro*, potato lectin, a hydroxyproline rich glycoprotein (Heilbronn *et al.*, 1995) Previously an extracellular protease from another isolate of the same bacterium was shown to be capable of degrading HRGPs from potato and tomato (Willis *et al.*, 1987). More recently, a metalloprotease has been isolated from *Xanthomonas campestris* that specifically degrades certain proline/hydroxyproline rich plant cell wall glycoproteins (Dow *et al.*, 1998).

Genetic analysis of proteases in a number of pathogens has been performed with mixed results. A protease deficient mutant of *Xanthomonas campestris* showed a considerable reduction in virulence on turnip leaves under certain inoculation conditions (Dow *et al.*, 1990). In contrast, a genetically defined protease mutant of *Cochliobolus heterostrophus* was still fully pathogenic (Walton, 1994). Similarly, mutants of *Erwinia chrysanthemi* lacking metallo-protease genes were reported to be unaltered in virulence (Dahler *et al.* 1990). However subsequent quantitative analysis was reported to have shown a statistically significant decrease in virulence (Walton, 1994). A UV induced protease mutant of *Cladosporium cucumerinum* was as pathogenic as the wild type on cucumber seedlings, although residual protease activity was detected during infection (Robertson, 1984). Similarly, protease mutants of *C. carbonum* showing reduced production of two trypsin-like proteases were indistinguishable in pathogenicity from wild type strains, although residual activity was detected *in vitro* and at least one other protease remained unaffected (Murphy and Walton, 1996). In contrast, a UV induced mutant of *Pyrenopeziza brassicae* lacking a cysteine protease was non-pathogenic. Protease activity and pathogenicity could be restored by

complementation with a single genomic cosmid clone (Ball *et al.*, 1991). Further evidence that proteases may play a role in plant pathogenesis was provided by the overexpression of an aspartic protease in *Cryphonectria parasitica* (Choi *et al.*, 1993). Strains containing multiple copies of an aspartic protease-encoding gene caused enhanced necrosis on chestnut bark and wood, suggesting that the overproduction of this protease had increased virulence.

1.4.7 Problems with disruption of genes encoding CWDE

Disruption of putative pathogenicity determinants frequently has no effect on disease, and cell wall degrading enzymes are no exception (Table 1.1). As discussed previously this may not always confirm that the enzyme plays no role in pathogenesis. It may be that no one single enzyme is responsible for pathogenesis and that only a cocktail of enzymes acting together are capable of degrading the wall (Knogge, 1996). There may be a second set of genes with similar function that are only produced *in planta*, latent gene(s) may be present, or the assay used to assess the virulence of the mutants may not be sensitive enough to measure the effect of the mutation. Alternatively the mutation may affect the long-term interactions between the host and pathogen or with other microorganisms on the leaf, which would require extended field-based assays. It is tenable that only when all modes of penetration, both enzymic and mechanical are lost will a reduction in pathogenicity be observed (VanEtten *et al.*, 1994).

Most common however is the presence of residual enzyme activity following gene disruption, which prevents assessment of the role of the enzyme in disease. This activity can often be attributed to the multiple isozymes of each CWDE produced by most fungi. Different isozymes of each CWDE may have different functions, including provision of nutrients during saprophytic growth, and they may be produced at different stages during infection (Cooper, 1983; Keon *et al.*, 1987). These apparent different isoforms may be a result of post-translational modifications, e.g. *Fusarium moniliforme* secretes four endopolygalacturonases derived from a single gene product (Capari *et al.*, 1993), multimer formation or

they may be true isoforms encoded by different genes. It is this latter situation that is most clearly responsible for residual activity following gene disruption and as a result some enzyme systems may be too complex to resolve genetically.

Attempts to address these problems have been made by multiple gene disruptions. A triple mutant of *C.carbonum* lacking three xylanase genes was created in an attempt to eliminate all residual activity (Apel-Birkhold and Walton, 1996). This was unsuccessful as residual activity remained and a fourth gene is currently being characterised. Only once all activity is lost, both *in vitro* and *in planta*, will it be possible to determine the importance of the enzyme in pathogenesis. However, if as Knogge (1996) suggests CWDE are released as a cocktail all required to work synergistically to achieve wall breakdown, multiple mutations in more than one enzyme may be required to reduce pathogenicity. Creating such mutations is now a key challenge. An indication of the possible success of this approach is the demonstration that pathogenicity can be reduced when secretion of CWDE is blocked either by mutation or chemical inhibition (Kotoujansky, 1987; Wattad *et al.*, 1995; Milling and Richardson, 1995).

The aim of this work is to study the CWDE of *S.nodorum* and assess their role in pathogenicity. In the absence of any evidence for a role for toxins in infection by this necrotrophic fungus, CWDE remain important potential pathogenicity determinants. This theory is supported by ultrastructural studies which show intercellular growth of the fungus and its ability to penetrate directly the plant cuticle (Zinkernagel *et al.*, 1988). This work aims to identify CWDE required by *S.nodorum* during infection of wheat. Detailed biochemical studies will be required to understand the possible roles played by these enzymes in disease and which of them may be important in pathogenesis. Cloning of the genes encoding these enzymes will enable their potential as novel fungicide targets to be tested by targeted gene disruption.

Chapter Two: Materials and Methods

All chemicals and reagents unless otherwise stated were supplied by Sigma (Dorset, UK).

2.0 Maintenance and culturing of *S.nodorum*

2.0.1 Fungal isolate

Stagonospora nodorum, isolate BS171 was obtained from AgrEvo UK Ltd, and was originally isolated from barley (Osbourn *et al.*, 1986).

2.0.2 Preparation and storage of spore stocks

5-7 day old plates of *S.nodorum* were flooded with sterile 0.02% Tween 20 in distilled water and spores released by scraping the surface of the plate with a 1ml pipette tip. This spore suspension was filtered through muslin to remove mycelial debris before centrifugation at 11,600 xg for 15min. Spores were resuspended in an equal volume of fresh Tween solution and then centrifuged again, as before, to wash the spores. Spores were resuspended in 20% glycerol at a concentration of 2×10^7 spores/ml and stored frozen as 1ml aliquots at either -70°C (long term) or -20°C (2-3months).

2.0.3 Growth of *S.nodorum* on agar plates

100µl of spore suspension (usually from frozen stocks, see 2.0.2) was spread over a Czapek-Dox V8 agar (CzV8CS, Newton and Caten, 1988) plate using a glass spreader. Plates were grown under continuous black light UV at 20°C for 5-7days to achieve sporulation. Czapek-Dox V8 agar contained per litre Czapek-Dox agar 45.4g, CaCO₃ 3.0g, Agar 10.0g, V8 juice 200ml, dH₂O 750ml and 50ml complete supplement (added after autoclaving). Complete supplement contained per litre, casamino acids 20.0g, mycological peptone 20.0g, yeast

extract 20.0g, adenine-HCl 3.0g, biotin 0.02g, nicotinic acid 0.02g, *p*-aminobenzoic acid, pyridoxine 0.02g, thiamine 0.02g.

2.0.4 Growth of *S.nodorum* in liquid culture

For the production of cell wall degrading enzymes the fungus was grown in shake culture. First, a fungal biomass was established in a complete liquid medium containing, per litre, 45.4g Czapek-Dox, 200ml filtered V8 juice and 100ml Complete Supplement (Newton and Caten, 1988). 2×10^7 spores were inoculated into 250 ml Erlenmeyer flasks containing 100ml of medium. The cultures were grown for 48hr at 24°C, 120rpm before filtration through muslin, then mycelium was washed with 50mM MES pH6.0 and transferred to a basal salts liquid medium for a 12h period of starvation; this medium contained per litre, 1g KH_2PO_4 , 2g NaNO_3 , 0.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 9.76g MES (50mM), (p.p.m), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2, ZnSO_4 1.0, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 0.02, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.02; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.02; pH was adjusted to pH6 with 1M NaOH prior to autoclaving. After 12 hours cultures were strained through muslin and washed with 50mM MES before transfer to inducing medium comprising basal salts medium (as above) plus 1% carbon source which was usually wheat cell walls.

2.0.5 Assessment of fungal growth

Mycelial dry weights could only be obtained from cultures grown in complete liquid medium due to the presence of insoluble wheat cell walls in subsequent cultures. Mycelia from cultures were harvested by filtration of cultures through a pre-weighed desiccated filter paper in a Buchner flask. Filters were then placed at 60°C in glass petri dishes until they reached a constant dry weight.

2.1 Growth and inoculation of plants

2.1.1 Plants

Wheat plants, varieties Hornet or Riband (for antibody infiltration work) which are moderately susceptible to *S.nodorum*, were supplied by Plant Breeding International, Cambridge. Plants were grown in a growth chamber at 20°C on a 12h light/dark cycle for 14 days for plant cell wall extraction or for ca. 7 days (until one leaf was fully extended) for inoculation. Plants were grown in trays of 35x24cm, seeds were evenly distributed across the tray.

2.1.2 Production of wheat cell walls

Wheat cell walls were prepared by roughly chopping 14 day old plants and grinding in liquid nitrogen with a pestle and mortar. Ground plant material was then soaked in 2.5 volumes of cold 0.1M phosphate buffer, pH6 before comminution in a Waring blender on high speed for 2-3min. The resulting suspension was filtered through 2 layers of muslin and the insoluble plant material resuspended in 2 volumes of cold phosphate buffer and allowed to stand for 5 min. This was repeated to a total of four washes in buffer and finally one in cold distilled water. Solvent extractions were performed with four washes of 1 volume chloroform:methanol (1:1v/v) and three washes of 2 volumes of acetone. All extractions were made at 4°C. Cell wall material was then left to dry overnight at room temperature then kept desiccated until required.

2.1.3 Whole plant inoculations

Spore suspensions in 0.02% Tween 20 of 5×10^5 spores/ml were prepared as for spore stocks but without washing of the spores. Seven day-old plants were inoculated with a hand held aerosol, sprayed at 90 degrees to the plant tissue until complete coverage had been achieved, but not run off. Plants were covered in polythene for 48 hours to maintain a high humidity and grown at 20°C with a 12h

light and dark cycle for 14 days for symptoms to develop. Control plants were sprayed with 0.02% Tween 20 alone. Germination tests were performed by placing the same spore suspension on clean glass slides in a humid chamber for 24h. The humid chamber was created by lining a sandwich box with damp tissue paper and placing two test tubes along its length, parallel to one another. Slides were then placed across the tubes and the chamber sealed with the lid. The chamber was placed in the plant growth chamber and germination assessed after 24h under a light microscope.

2.1.4 Detached leaf inoculations for western blot analysis

In order to generate infected plant material more quickly than the 14 days required by whole plant inoculations, a more rapid method for producing infected plant material was used, as described by Dr.C.Caten, Birmingham University (pers. comm.). Leaves were picked from seven day-old wheat plants and dipped into 1×10^5 spores/ml in 0.02% Tween 20. Inoculated leaves were then laid onto benzimidazole-tap water agar plates (0.5% agar, 150 μ g/ml benzimidazole made up in tap water), sealed with parafilm and incubated at 20°C under a 12h light/dark cycle and disease symptoms allowed to develop. Using this method symptoms developed in half the time of that required by spraying whole plants. Typically, the severe symptoms apparent on whole plants after 14d occurred by day 7 on inoculated, detached leaves. Samples of leaves were taken at 24h, 48h, 4 days and 7 days after infection, corresponding to no symptoms, chlorotic spots appearing, necrotic spreading lesions and on day 7 when leaves were moribund.

2.1.5 Extraction of enzymes from plant material

Plant material was ground in liquid nitrogen and the powder suspended in cold extraction buffer (5ml/g), comprising 50mM sodium phosphate buffer pH6, 5mM dithiothreitol (to prevent oxidation), 0.2M NaCl (to desorb proteins from cell walls) and 5% (w/v) polyvinyl pyrrolidone (to adsorb phenols). Following shaking on ice for 15 min, extracts were centrifuged at 4,000g for 15 minutes at

4°C and the supernatant re-spun at 23,000g for 15 min. Extracts were dialysed overnight against several changes of 50mM citrate buffer pH 6 before concentration in a solution of polyethyleneglycol 20,000 Mr to a final concentration equivalent to 1g of plant tissue per ml of extract.

2.1.6 Effect of inhibitors on disease symptoms

Two cm sections of 10 day-old wheat plants were taken 3cm from the tip and either end embedded in benzimidazole tap water agar to form a bridge. A single leaf section was placed in each 9cm petri dish. Spore suspension containing 10^6 spores ml^{-1} in 0.02% Tween 20 was mixed with an equal volume of various protease inhibitors. All inhibitors were prepared in water except pepstatin which was prepared as a 1mM stock in DMSO. These and controls (0.02% Tween 20) were incubated at room temperature for 1 hour before a 5 μ l droplet of each suspension was inoculated onto the leaf bridges, ten leaves per treatment. These dishes were then sealed with parafilm and incubated at 20°C for 7 days with a 12h light and dark cycle. Macroscopic symptoms were assessed periodically and a disease score given, based on:

- 0 - no disease
- 1- water soaking
- 2 - chlorosis
- 3 - necrotic spots in a chlorotic region
- 4 - necrotic lesion
- 5 - spreading necrotic lesion

The effect of these inhibitors on spore germination *in vitro* was assessed following incubation of the spore suspensions overnight on glass slides in a humid chamber (method as described previously in section 2.1.3).

2.1.7 The effect of inhibitors on spore germination *in vivo*

The effect of protease inhibitors on spore germination *in vivo* was assessed at 14 and 24h after inoculation. Inoculations were set up as described in section 2.1.6 and at each time point three leaf bridges were removed for each treatment. These bridges were placed onto damp tissue paper soaked in ethanol:acetic acid 1:1 for 1 day to remove chlorophyll. Leaves were then placed on damp tissue paper soaked in lactic acid:glycerol:H₂O 1:1:1 for 1 day. Spores were visualised by staining with lacto-phenol cotton blue (0.05%) and germination assessed under a light microscope.

2.1.8 Infiltration of antibody solutions into wheat leaves

Wheat leaves were infiltrated with antibody solutions using a 10ml plastic syringe, modified as follows. The tip was heat sealed in the flame of a bunsen burner and a hole cut at the 4ml mark with a hacksaw (Fig.2.1)

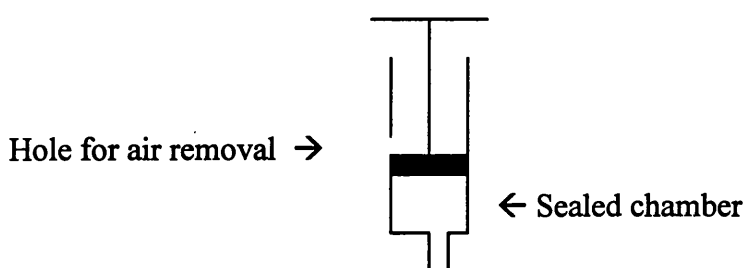


Fig.2.1. Modified 10ml syringe used for infiltrating antibody solutions into wheat leaves.

Five wheat leaves were then placed in the syringe and 5ml of antibody solution (10, 1 or 0.1%) or distilled water (control) was added. The plunger was then replaced and pushed down until all of the air and excess liquid had been expelled from syringe via the hole at the 4ml mark. This allowed a constant pressure to be applied to the leaves for 15 seconds which forced the solutions into the leaves. Leaves were removed, briefly dried on tissue before the base of each leaf was placed in distilled water for 1.5h to allow transpiration to remove excess water

from the leaves. Leaf segments were then embedded at either end in benzimidazole tap water agar prior to inoculation with a 5µl droplet of a spore suspension. Spore suspensions were at 1×10^3 , 10^4 , 10^5 , 10^6 and 10^7 spores/ml. Ten leaves for each antibody and spore concentration were inoculated and symptoms assessed after 7 days.

2.1.9 Culture of tritordeum suspension cells

Tritordeum embryogenic suspension cells were kindly supplied by P.A. Lazzeri Rothampstead experimental station, Harpenden, Herts. Cells were grown in a medium containing per litre: 100ml macrosalts, 1ml microsals, 10ml FeNaEDTA, 5ml Vitamins+inositol, 1.5g glutamine, 0.3g proline, 0.2g asparagine, 60g maltose, 2ml of 2,4-D (1mg/ml). Macrosalts contained, per litre: 2.5g NH_4NO_3 , 15.0g KNO_3 , 2.0g KH_2PO_4 , 3.5g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 4.5g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Microsals contained per litre: 10.7g $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 6.2g H_3BO_3 , 5.8g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8g KI, 0.25g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.025g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Vitamins plus inositol contained per litre: 40g inositol, 2g thiamine, 0.2g pyridoxine, 0.2g nicotinic acid, 0.2g Ca-pantothenate, 0.2g ascorbic acid.

The final medium was adjusted to pH 5.7 and filter sterilised. Cells were cultured at a starting density of 4g per 10ml of medium, shaking at 70 rpm at 25°C in the dark and subcultured into fresh medium every seven days.

2.2 Enzyme assays

Enzyme activities were converted to nKats for all assays except viscometry and azo dye and remazol brilliant blue dye-linked substrates. One katal corresponds to the conversion of one mole of substrate per second, hence:

$$1\mu\text{mol} \times \text{min}^{-1} = 16.67 \text{ nkats}$$

2.2.1 Nelson-Somoygi reducing sugar assay

Reducing sugar released by the action of xylanase, polygalacturonase, cellulase and laminarinase on their respective substrates was detected using the Nelson-Somogyi reducing sugar assay (Nelson, 1944). Reactions were performed in triplicate with two time zero controls (to measure background sugar). 100 μ l of test enzyme solution was added to a clean glass test tube and to time zero controls to which 500 μ l of stopping reagent was also added (Stop reagent was a mixture of reagents A and B at 25:1 prepared 30min before use. Reagent A: g/l, Na_2CO_3 25g, Sodium potassium tartrate 25g, NaHCO_3 20g, filtered. Reagent B: 15% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ with 1 or 2 drops of H_2SO_4 added per 100ml). Citrate buffer controls were also included in place of enzyme. Substrate solution was pre-incubated at 37°C for 10 min before 500 μ l was added to test samples. Reactions were incubated at 37°C for 30min or 2h for cellulase (which has an insoluble substrate). Substrates, all dissolved or suspended in 50mM citrate pH 5, were: 0.1% Birch wood xylan, 0.1% polygalacturonic acid, 0.1% laminarin and 1% Whatman no.1 filter paper (homogenised) for xylanase, polygalacturonase, laminarinase and cellulase assays respectively. Reactions were stopped by the addition of 500 μ l of stopping reagent and tubes capped before transfer to a 95°C water bath for 30 min. Tubes were then allowed to cool to room temperature before the addition of 500 μ l of Nelson reagent C (25g of ammonium molybdate was dissolved in 450ml of distilled water in a clean acid washed glass bottle before addition of 21ml of conc. H_2SO_4 . Dissolved separately was 3g of $\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$ in 25ml of distilled water before addition to the ammonium molybdate solution. The reagent was then incubated in the dark at 37°C for 24h).

Tubes were then vortexed to mix and left for at least 2h for the colour to develop. 200µl was then removed to a microtitre plate and the absorbance read at 595nm on a Dynatech MR5000 plate reader against citrate buffer blanks. If necessary, samples were transferred to 1.5ml eppendorf and centrifuged at 11,600 xg for 10 min to remove undigested substrate before reading the supernatant.

The concentration of substrate released was measured from a standard curve and the enzyme activity converted into nkats. Standard curves were produced using known concentrations of the sugar monomers xylose, galacturonic acid and glucose in the range 0-1mM. Total sugar was measured by adding 500µl of stopping reagent to 1ml of sugar standard followed by incubation at 95°C for 30min. After cooling 500µl of Nelson C was added and absorbance determined as described above.

2.2.2 Remazol Brilliant Blue xylanase assay

Xylanase activity was assayed spectrophotometrically by measurement of enzyme-released dyed fragments soluble in the presence of organic solvents which precipitate the original chromogenic substrate, Remazol brilliant blue dyed xylan, as developed by Biely *et al.* (1985). Reactions were performed in duplicate and a boiled enzyme control was included for each sample. 180µl of 0.1% Remazol Brilliant Blue dyed xylan in 50mM citrate pH5 was added to 20µl of enzyme solution, boiled enzyme or citrate blank. Reactions were incubated at 37°C for 1h or 20h for *in vitro* and *in vivo* samples respectively. Reactions were stopped by the addition of 500µl of 96% ethanol. Tubes were then mixed and left to stand for 5-30 min before centrifugation at 11,600 xg for 2 min. 200µl of the supernatant was then removed to a microtitre plate and the absorbance read at 595nm on a Dynatech MR5000 plate reader against the citrate blanks.

2.2.3 Protease assays

2.2.3.1 Azocasein

General protease activity was assayed spectrophotometrically by measuring the release of red azo-dyed low molecular weight peptides from azocasein at pH 5.0 and 8.0. Reactions were performed in duplicate with duplicate boiled controls. Reaction mixtures contained 100µl of 3% azocasein made up in either 50mM citrate pH 5.0 or Tris-HCl pH 8.0, 350µl of 50mM citrate pH5 or Tris-HCl pH 8.0 and 50µl of test sample, boiled control or buffer blank. After incubation at 37°C for 3h the reaction was stopped by the addition of 500µl of 20% TCA. The solutions were then vortexed to mix and centrifuged at 11,600 xg after which 200µl of supernatant was removed to a microtitre plate and the absorbance read at 340nm against buffer blanks.

2.2.3.2 Trypsin assay

Trypsin-like protease activity was measured spectrophotometrically against the nitroanilide substrate, Benzoyl-Phe-Val-Arg-Nitroanilide. Reaction mixtures contained 50µl of enzyme solution, 100µl of 50mM Tris-HCl pH8 and 50µl of 2mM substrate made up in DMSO. Nitroanilide release was monitored at 405nm over 15minutes. Enzyme substrate specificity was investigated under the same conditions using the substrates Suc-(Ala)₂-Pro-Phe-NA, CBZ-Gly-Gly-Leu-p-NA, BZ-Phe-Val-Arg-NA, Ben-Pro-Phe-Arg-NA, D-Val-Leu-Arg-NA, Ben-DL-Arg-NA and D-Val-Phe-Lys-NA.

2.2.3.3 Aspartic protease

Aspartic protease activity was assayed by adding 200µl of culture filtrate to 600µl of 1% BSA in 50mM sodium citrate, pH3.2. After 2h at 37°C 400µl of 10% (w/v) trichloroacetic acid was added to precipitate undigested protein and the tubes stored on ice for 10min. Following centrifugation at 11,000 xg for

10min the absorbance of the supernatant was read at 280nm and corrected for background using a time-zero control in which trichloroacetic acid was added prior to the enzyme.

2.2.4 Pectin Lyase

Pectin lyase was assayed spectrophotometrically by measuring $A_{240\text{nm}}$ in a Cecil spectrophotometer with quartz cuvettes. The reaction mixture contained 700 μl of 0.25% pectin in 50mM Tris-HCl pH 9.0, 1mM CaCl_2 (final concentration) and 300 μl of enzyme test solution. The reaction mixture was incubated at 37°C and absorbance measured at time zero, 15 min and 30 min for two test and two boiled controls of each sample.

2.2.5 Glycosidases

Glycosidase activities were assayed by measuring the increase in $A_{410\text{nm}}$ from the release of *p*-nitrophenol from their respective *p*-nitrophenyl-linked substrates. These were α -L-arabinofuranoside, β -D-galactoside, β -D-glucoside, α -D-glucuronide, β -D-xylopyranoside and acetate. All glycosides were dissolved in 50mM citrate buffer at pH5.0 at 5mM. Each reaction mixture consisted of 180 μl of buffered glycoside solution and 20 μl of enzyme test solution or boiled control. Reactions were performed in duplicate, with duplicate boiled enzyme controls and buffer blanks. Reactions were stopped after incubation at 37°C for 30min or 20h for liquid cultures and plant extracts respectively by the addition of 500 μl of 1M NaHCO_3 . After 30min absorbance was read at 410nm against citrate buffer blanks. A calibration curve of *p*-nitrophenol was made in the range 0-50 μmoles .

2.2.6 Carbohydrate assay

Total carbohydrate was determined by the method of Dubois *et al.* (1956). 10 μl of 80% phenol (w/w) was added to 0.4ml of aqueous sample and 1ml of concentrated sulphuric acid was added and the sample mixed. Following

incubation at room temperature for 10min the mixture was cooled in a water bath for 10min and the absorbance read at 485nm. Glucose (2-15 μ g) was used to produce a standard curve.

2.3 Enzyme characterisation by isoelectric focusing

2.3.1 Gel Isoelectric focusing

Isoelectric focusing was performed with precast polyacrylamide gels and pI markers in the pH range 3-9.5 (Pharmacia, Sweden). These were pI in brackets: amyloglucosidase (3.5), methyl red (dye) (3.75), soybean trypsin inhibitor (4.55), β -lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic band (6.85), horse myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.30). Samples were electrophoresed at 4°C 30W constant power for 1.5h as per manufacturer's instructions with 1M NaOH and 1M H₃PO₄ as the cathode and anode solutions respectively. Following separation, proteins were fixed for 30-60min in fixing solution (29g TCA, 8.5g sulphosalicylic acid, dissolved in distilled water and made up to 250ml). After fixing, gels were briefly washed for 5min in destain (25% ethanol, 8% acetic acid) then stained in 0.5% Coomassie blue R-250 in 50% methanol, 7.5% acetic acid for a minimum of 15 minutes. Gels were subsequently destained in several changes of destain solution until the background became clear. Protein pIs were estimated by measuring the distance travelled from the cathode compared to the known proteins in the pI marker.

2.3.2 Protease overlay detection in IEF gels

Protease activity was detected in replica IEF gels by overlaying gels with unprocessed gelatin-coated X-ray film incubated previously for 10min in water (Abraham and Breuil, 1996). Protease activity was observed as a blue clearing

zone on the film due to degradation of the gelatin layer after ca. 30min at room temperature.

2.3.3 Xylanase overlay detections in IEF gels

2.3.3.1 RBB-dyed xylan overlay

Xylanase activity was detected in replica IEF gels using an RBB-dyed xylan overlay gel (this was the substrate used to detect xylanase activity by assay). The gel was prepared (adapted from the method of Biely *et al.*, (1985b)) by dissolving 150mg of RBB-dyed xylan in 10ml of dH₂O at 60°C and subsequently mixing this with 20ml of a hot 1.5% (w/v) agarose solution in 0.05M citrate pH5. This mixture was then poured between SDS-PAGE plates (Biorad) with 0.75mm spacers. The RBB-gel was overlaid onto the IEF gel and the two gels incubated together at room temperature until clearing zones appeared, after which the overlay gel was destained in 96% ethanol for 3-20h.

2.3.3.2 Oat spelt xylan overlay

Oat spelt xylan overlay gels were also tried, prepared as above but using different methods for detecting clearing zones i.e.washing in 95% ethanol (Royer and Nakas, 1990) or by washing with 1M NaCl followed by staining with 0.1% Congo red (binds to β -1,4-glucans) for 30min followed by further washes in 1M NaCl (MacKenzie and Williams, 1984).

2.3.4 Preparative Rotofor™ IEF

50ml of 48h cell wall grown culture filtrate was extensively dialysed against dH₂O before the addition of 1% ampholytes (BioRad), pH range 3-10. Culture filtrate was then loaded into a Rotofor™ focusing chamber (BioRad), assembled as per the manufacturer's instructions using 0.1M NaOH and 0.1M H₃PO₄ as cathode and anode solutions respectively. Separation of the proteins then took

place at 12W constant power at 4°C until the voltage stabilised, typically after 4 hours. Fractions were then harvested under vacuum and the pH, protein content and enzyme activity in each fraction determined.

2.4. Purification of SNP1

2.4.1. Ammonium sulfate precipitation

After 48h growth in inducing medium with 1% wheat cell walls, 500ml of culture filtrate was harvested by filtering through two layers of muslin. Proteins were concentrated by precipitation with ammonium sulphate to 80% saturation, by the gradual addition of ammonium sulphate stirred at 4°C. The precipitated protein was centrifuged at 14,000g for 30min at 4°C and the supernatant discarded. The protein pellet was resuspended in 50ml of 25mM MES pH6.0 and dialysed against several changes of similar buffer for 24h to remove the ammonium sulphate. Dialysed protein extract was then centrifuged at 10,000xg for 15 min to remove any remaining debris and the supernatant filtered through a 0.2µm nitro-cellulose filter.

2.4.2 Partial purification of an alkaline protease

10ml of this concentrated fungal culture filtrate was loaded at 1ml/min onto a 10ml S-sepharose (fast-flow) cation exchange column which had previously been equilibrated with 25mM MES pH6.0. Unbound protein was washed through the column with 25mM MES pH6.0 in 10 column volumes at 1ml/min. Bound proteins, including the protease were eluted over a 0-250mM NaCl gradient, over 10 column volumes and fractions assayed for protease activity using azocasein at pH 8.0.

2.4.3 Purification of a trypsin-like protease (SNP1)

To purify the protease to homogeneity two steps of cation exchange chromatography were used. In the first, 10ml of the concentrated fungal culture filtrate extract was applied to a 5ml cation exchange column (High trap™ SP sepharose Pharmacia Biotech) previously equilibrated in 25mM MES pH 6.0, run on a Pharmacia FPLC system. Unbound protein was washed from the column with the same buffer in 10 column volumes at a flow rate of 1ml/min. Bound protein was eluted over a 0-175mM NaCl gradient in 25mM MES pH6 at 1ml/min over 10 column volumes and detected using an on-line UV-recorder (LKB). Fractions containing trypsin-like protease activity (detected by assay with Benzoyl-Phe-Val-Arg-NA) were pooled and buffer exchanged using PD-10 columns (Pharmacia) into 50mM HEPES-MES-Na Ac at pH 4.5. The active FPLC peak was applied to a 0.8ml high resolution cation exchange column (HS20 Poros Boehringer Mannheim) previously equilibrated in 50mM HEPES-MES-NaAc at pH4.5, run on a Biocad™ Sprint™ Perfusion Chromatography System. Unbound proteins were washed out in this same buffer in 10 column volumes at 8ml/min and bound proteins eluted over a 0-200mM NaCl gradient, again in the same buffer, over 20 column volumes. Fractions were then assayed for trypsin-like protease activity and electrophoresed on SDS-PAGE to determine purity.

2.5 Protein electrophoresis and western blotting

2.5.1 Protein assay

Protein determinations were carried out using the dye binding method (Bradford, 1976) in which Bio-Rad reagents were used with BSA as standard.

2.5.2 SDS PAGE

Denaturing SDS-PAGE was performed following the methods of Laemmli (1970) using the BioRad Mini-Protean™ SDS-PAGE system. 12% polyacrylamide gels were routinely run and these contained in the separating gel, per gel: 2.05ml 30% acrylamide, 1.25ml 1.5M Tris-HCl pH8.8, 1.665 ml MQ H₂O, 50µl 10% ammonium persulphate, 37.5µl 10% SDS, 5µl TEMED. In the stacking gel, 375µl 30% acrylamide, 625µl 0.5M Tris-HCl pH6.8, 1.5ml MQ H₂O, 25µl 10%APS, 25µl 10%SDS, 2.5µl TEMED. Running buffer contained per litre 0.3% Tris, 14% glycine and 1% SDS at pH 8.3 using HCl. Samples were prepared in loading buffer containing 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, 0.0625M Tris-HCl pH 6.8 and boiled for 3min before loading. Samples were electrophoresed along side BioRad broad range molecular weight markers. These were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and α-lactalbumin (14.5 kDa). Gels were electrophoresed at 100 V until the dye front reached the bottom of the stacking gel, and then at 150V until the dye front had run off the bottom of the gel. To visualise protein bands the gel was stained with 0.5% Coomassie blue R-250 in 50% methanol, 7.5% acetic acid for a minimum of 15 minutes. The gel was then destained in several changes of 45% methanol and 9% acetic acid.

Gels used for blotting proteins for N-terminal sequencing were cast the day before use to reduce the presence of unpolymerised acrylamide, which can block

N-termini. Electrophoresis of these gels also included 2mM mercaptoacetic acid (thioglycollic acid) in the upper running buffer. This mobile thiol runs ahead of the protein and scavenges N-blocking free-radicals which may be present in unpolymerised acrylamide.

2.5.3 Preparation of antibodies

The method for antibody production was adapted from St.Leger *et al.* (1996a). Partially purified preparations of Xyl1 and SNP1 were electrophoresed using SDS-PAGE to achieve separation of the protein components. After electrophoresis, the gel was washed briefly in water and stained for 10min with 0.05% Coomassie blue prepared in water. The gel was washed repeatedly in water, and the stained protein bands corresponding to Xyl1 and SNP1 excised. The gel slice containing approximately 50µg of Xyl1 or SNP1 was then lyophilised, ground into a powder and resuspended in 800µl of water. This was then mixed with 500µl of Freund's complete adjuvant which was injected subcutaneously into a rabbit. This was followed by three subsequent injections, 14 days apart where Freund's complete adjuvant was substituted by Freund's incomplete adjuvant. One week after the final injection the rabbits were exsanguinated and the serum was collected and stored at -20°C.

2.5.4 Electroblothing

Following separation of proteins by SDS-PAGE, gels were equilibrated with agitation for 30 min in transfer buffer; 10mM CAPS, 5mM dithiothreitol (DTT) and 10% methanol, pH 11. For N-terminal sequencing, the gels were blotted onto ProBlott® membranes (Applied biosystems) or for western analysis Hybond C-nitrocellulose (Amersham). ProBlott® membranes were prepared by immersing briefly in methanol before equilibration for 10min in transfer buffer. Hybond C membranes were prepared by equilibration for 30min in transfer buffer. Membranes and gels were assembled as described in the manufacturers instructions and electroblotted in transfer buffer for 1h at 350mA using the Mini-

Protean™ electroblotting system (BioRad). Once transfer was complete membranes for western analysis were processed as described below. ProBlott membranes for N-terminal sequencing were first washed in water for 10min, all steps with agitation. Membranes were then stained with 0.1% Coomassie Blue R-250 in 50% methanol/1% acetic acid for 5min and destained in several changes (minimum of 3) of 50% methanol for 5 min each. Membranes were finally washed in dH₂O for 3x10min thorough air-drying.

2.5.5 Western Blot Analysis

Immunodetections were performed as follows, all steps with shaking. The membrane was blocked in 10ml of Tris buffered saline (TBS; 20mM Tris-HCl, 0.5M NaCl pH 7.5) containing 5% milk powder for 1h. Blots were washed twice for 5min in 20ml of TBS+0.05% Tween 20 (TBST) before incubation with primary antibody diluted in 10ml of TBST containing 3% milk powder for a minimum of 1h. To remove unbound antibody blots were then washed 4 times in 20ml TBST for 5min each before incubation with the secondary antibody for 1h. This was a goat anti-rabbit antibody conjugated to alkaline phosphatase, diluted 1:10,000 in TBST+3% milk powder. Following binding of the secondary antibody, blots were washed four times for 5min each in 20ml of TBST with one final wash for 5min in 20ml of TBS. Detection of the bound antibody conjugate was performed by the addition of 1ml of alkaline phosphatase substrate BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and the colour allowed to develop. To stop colour development blots were washed repeatedly in distilled water.

2.6 Molecular Methods

2.6.1 Preparation of genomic DNA

DNA was isolated by a method adapted from Raeder and Broda (1985). Mycelia was obtained from *S.nodorum* grown for 72h in complete Czapek-Dox medium as described in section 2.0.4. Cultures were harvested by filtering through four layers of sterile muslin then squeezed dry. Mycelium was then removed to aluminium foil, flash frozen in liquid nitrogen and stored at -80°C until required. Mycelium was ground to a powder in liquid nitrogen with a pestle and mortar and added to 10ml of fungal extraction buffer (200mM Tris-HCl pH 8.5, 250mM NaCl, 25mM EDTA pH8.0, 0.5% w/v SDS) in a FEP (Fluorinated ethylene propylene, solvent resistant) tube. 10ml of phenol:chloroform (7:3), equilibrated with fungal extraction buffer, was added and the tube shaken gently for a few minutes to remove protein. Following centrifugation at 10,000g at 4°C for 15min the aqueous phase was extracted again with phenol:chloroform before being removed to a clean FEP tube and RNA removed by digestion with DNase free RNase A (10µg/ml) for 1h at 37°C. RNase A and proteins were removed by mixing with an equal volume of chloroform: isoamylalcohol (24:1) for 15min followed by centrifugation at 10,000xg at 4°C for 30min. The aqueous phase was then removed to an oakridge tube and DNA precipitated by the addition of 0.6 volumes of isopropanol. DNA was pelleted by centrifuging at 7000xg at room temperature for 15 min. The DNA pellet was washed twice with 70% ethanol to remove traces of salt, before air drying at room temperature for 30min. Finally, the DNA pellet was redissolved in 2ml of TE pH8 (10mM Tris-HCl pH8.0, 1mM EDTA).

2.6.2 DNA restriction and agarose gel electrophoresis

1-10 µg of DNA was digested with 10 units of restriction enzyme in the appropriate buffer at 37°C for 1h. Loading buffer (6x gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (v/v) glycerol) was added and

digestion products separated by electrophoresis on agarose gels (0.7-1.5%), in 0.5xTBE (containing per litre: 5.4g Tris base, 2.75g boric acid, 2ml 0.5M EDTA pH8.0). Ethidium bromide was incorporated into the gel at a concentration of 0.5µg ml⁻¹ and DNA visualised under UV (320nm) on a transilluminator.

2.6.3 PCR Primers

For generation of a PCR product encoding the *try1* gene from *Metarhizium anisopliae* the specific primers, ATGGAGCTCAAGTCCCTACT (forward) and CAAGTGGCTCTTGATGAACT (reverse) were designed, based on the published sequence (Smithson *et al.*, 1995).

For generation of a PCR product encoding the SNP1 gene of unknown sequence two degenerate primers were designed based on an alignment of three fungal trypsin sequences (Fig.2.2). Primers Try1 and Try2, were designed to the second half of the N-terminus and the trypsin active site respectively.

Primer sequences were:

Try1: GCN GGN GAN TWY CCN TTY ATH GT

Try2: GGN CCN CCN SWR TCN CCY TGR CA

Subsequent primer sets used were Tryp1 and Tryp2, specific primers based on the PCR product sequence generated by Try1 and Try2. An additional primer based on the SNP1 trypsin N-terminus was also used, sequences below.

Tryp1: TTC TGC GGT GGT TCG CTG CT

Tryp2: TTG GAG TTG CAA GTG GAG CG

N-term: ATH GTN GGN GGN ACN ACN GC

<i>Fusarium oxysporum</i>	MVELKSLLAV	YL.ALVAPLA	AAAPQEIPN.	30
<i>Cochliobolus carbonum</i>	.MRFQSMITA	ALPALVLSAP	TPQWDDVPED	
<i>Metarhizium anisopliae</i>	.MKFASVV..	..P.LCAAPL	AAARPASNAV	

	Try1→			80
F.o.	.IVGGTSASA	GDFPFIVSIS	RNGGPWCGGS	LLNANTVLTA
C.c.	SIVGGTTAAA	GEYPFIVSIQ	LGGRHNCGGT	LINGNTVVTA
M.a.	FIVGGSPAAA	GEFPPFIVSTL	LNGRHWCGGV	LLNANTVLTA
				130
F.o.	G....FQIRA	GSLSRTSGGI	TSSLSSVRVH	PSY..SGNNN
C.c.	GSINNVAVRV	GSLSANS GGQ	VIKVS KIIH	PSYQASTSNN
M.a.	S.....QVRA	GSLAHASGGV	VANISSITPH	PKYE..GLGY
				180
F.o.	IPSGGNIGYA	RLAASGSDPV	AGSSATVAGW	GATSEGGSSST
C.c.	VTAGGNIGFA	SLAASGSDPA	SGSTSSVAGW	GATREGGGAN
M.a.	IEANGTI..V	RHIARGSDPV	GGADATVAGW	GDL.EYAGQA
				230
			← Try2	
F.O.	IVSRATCRAQ	YG TSA....I	TNQMFCAGVS	S.GGKDSCQG
C.c.	IVARSTCVSN	YNAVG..LTV	TTNMVCAGVT	A.GGRDSCQG
M.a.	VVDRATCSAA	YQAIPNMPNI	TDAMFCAGLK	E.GGQDACNG
				270
F.o.	DSS.NTLIGA	VSWGNGCARP	NYSGVYASVG	ALRSFIDTYA
C.c.	DAN.KTLIGV	VSWGTCARP	NLPGVYSRVG	TLRSFIDQNA
M.a.	DTETRVLIGV	VSWGKCAAP	NAYGVYTRLG	ADIEFIKSHL

Fig.2.2. Alignment of three fungal trypsin protein sequences from *Fusarium oxysporum* (Rypniewski *et al.*, 1993), *Cochliobolus carbonum* ALP1 (Murphy and Walton, 1996) and *Metarhizium anisopliae* try1 (Smithson *et al.*, 1995). The sites at which degenerate primers are based are labelled Try1 and Try2.

2.6.4 PCR Isolation of the *try1* gene and *SNP1*

PCR amplification of a 560 bp region of *SNP1* using primers Try1 and Try2 from *S.nodorum* and 928bp of the *try1* gene from *Metarhizium anisopliae* was performed as follows: In 25µl reaction volume 1xPCR reaction buffer (Bioline); 0.2mM each of dATP, dCTP, dTTP and dGTP; 1.5mM MgCl₂; 1 U *Taq*

polymerase (Bioline); 50pmol oligonucleotide primer; 50ng of genomic DNA. PCR reactions were performed in an MJ Research Inc. PTC-100™ Programmable Thermal Controller under the following conditions: 1min of denaturation at 94°C; 35 cycles of 1min denaturation at 94°C, 1min of annealing at 55°C and 1min of primer extension at 72°C; 5 min of primer extension at 72°C.

2.6.5 Southern blotting and hybridisation

5µg of genomic *S.nodorum* DNA was digested with *Bam*HI, *Eco*RI and *Hind*III and digestion products separated overnight in a 0.7% agarose gel. Following electrophoresis agarose gels were soaked in 0.25M HCl for fifteen minutes to depurinate the DNA, denatured in 0.5M NaOH, 1.5M NaCl for thirty minutes and then neutralised in 1M Tris-HCl (pH 7.0), 1.5M NaCl for thirty minutes. DNA was transferred to nylon membrane, by a method modified from that described by Southern (1975), by placing the gel on six sheets of 3MM Whatman paper soaked with 10xSSC (20x SSC containing per litre: 175.3g NaCl, 88.2g Nacitrate, pH7) covering with a nylon membrane (Amersham), followed by 2 pieces of 3MM Whatman paper on top of which was placed a 5cm stack of paper towels. A kilogram weight was placed on top to ensure a good contact between the towels and the membrane. Transfer of DNA took place overnight, after which the membrane was washed in 2xSSC to remove traces of agarose, air dried and DNA fixed to the membrane by exposure to UV illumination (320nm) for five minutes. Membranes were stored at -20°C prior to hybridisation.

Membranes were prehybridised at a temperature dependant on the probe, 50°C (*tryl* gene) and 65°C (*SNP1* PCR product) in 100ml of prehybridisation solution (0.265 M Na₂HPO₄·2H₂O (pH7.2), 7% SDS, 1% skimmed milk powder) for at least 1h before the addition of ³²P labelled PCR product. PCR products used as probes were excised from an agarose gel and the DNA purified using a Pharmacia Biotech Gel-band purification kit. 25ng of probe DNA was labelled using α-³²P dCTP and the Ready-To-Go DNA labelling kit (Pharmacia). Probe

was added to filters and hybridised for a minimum of 16h at the appropriate temperature. Filters for the *tryI* heterologous Southern were washed three times in 2xSSC 0.1% SDS for fifteen minutes at 50°C. Filters for the *SNP1* PCR product Southern were washed at 65°C in 2xSSC 0.1% SDS for 15min, 1xSSC, 0.1% SDS for 15min with the most stringent wash for 15min in 0.5xSSC, 0.1% SDS. Filters wrapped in Saran wrap were placed in contact with X-OMAT LS film (Kodak) in the presence of an intensifying screen for two to six days at -70°C before developing.

2.6.6 Plating cells

100ml of LB supplemented with 1ml of 10% maltose and 1ml of MgSO₄ was inoculated with a single colony of *E.coli* strain LE-392 and grown overnight at 37°C with shaking at 200rpm. Cells were chilled on ice and stored at 4°C for up to ten days.

2.6.7 Heterologous library screen with the *tryI* gene

The PCR product of 928bp encoding the *M.anisopliae tryI* gene was used to screen a genomic *S.nodorum* lambda library. The lambda library used for screening was *S.nodorum* isolate LAW, constructed in lambda GEM11 (Promega) kindly donated by Dr.John Hargreaves at Long Ashton Research Station, Bristol. The library was prepared by a partial digest of DNA with *Sau3A* I, which was size separated by electrophoresis and fragments of 14-23kb ligated into the vector following the manufacturer's instructions.

An aliquot of the library, corresponding to 20,000 plaque forming units per plate was incubated at 37°C for 30min with 1.5ml of plating cells. Phage suspension was mixed with 50ml of molten LB agarose (0.8% w/v, cooled to 47°C) and poured onto a 27cm square plate containing set LB agar (2% w/v). Top agar was allowed to set before inversion and incubation at 37°C overnight. The plate was placed at 4°C for 1h after the number of plaque forming units (approx. 20,000)

had been confirmed to be present. Two nylon filters (Amersham) were cut to the size of the plate and the first was placed onto the surface of the agar, marked asymmetrically with needle and ink, and left in contact with the plate for 1min before being replaced with the second filter. This replicate filter was also marked, as previously, but was left on the plate for 5min before being removed. Once removed, the DNA on the filters was denatured by placing, DNA side up, on 3mm Whatman filter paper soaked in 0.5M NaOH, 1MNaCl for 5min. Neutralisation was performed on paper soaked in 0.5M Tris-HCl (pH7.4), 1.5M NaCl for 5min followed by paper soaked in 20xSSC. DNA was fixed to the nitrocellulose by treatment with ultraviolet light for 5min. Filters were hybridised and washed as described for Southern blotting. Filters wrapped in Saran wrap were placed in contact with X-OMAT LS film (Kodak) in the presence of an intensifying screen for two to six days at -70°C. The X-ray film was developed, and positively hybridising plaques on both filters identified by alignment of duplicate autoradiographs. The area containing each positive plaque was removed from the agar plate using a corkborer and placed in 3ml of SM buffer overnight at 4°C (100mM NaCl, 8mM MgSO₄·7H₂O, 50mM Tris-HCl pH7.5, 0.01% w/v gelatin) containing one drop of Aristar chloroform. 10 positively hybridising plaques were selected in this way and four dilutions (1, 1/10, 1/100, 1/100) of each eluted phage was tested on a secondary round of screening. 10µl of eluted phage dilution was added to 100µl of plating cells and incubated at 37°C for 30min. 4ml of top agarose (0.8% w/v, cooled to 47°C) was added to each dilution and poured onto LB agar plates (9cm diameter) and allowed to set before incubation overnight at 37°C. Secondary screening proceeded as above with duplicate filters. Six positives were selected based on their hybridisation on both duplicate filters. Positives were selected as single plaques and added to 500µl of SM buffer and eluted overnight at 4°C.

A high titre plate lysate stock for each positive lambda clone was prepared by plating 20µl of each phage suspension in 100µl of plating cells as described above. After overnight incubation at 37°C the top agar was scrapped off into 5ml

of SM buffer and left overnight at 4°C to elute. Agar was removed by centrifugation.

2.6.8 Lambda DNA preparation

1ml of the high titre stock solution for each positive lambda clone was used to infect 200ml of LE-392 cells at A_{600nm} of 0.6 in NZYCM (per litre: 10g NZ amine, 5g NaCl, 5g bacto-yeast extract, 1g casamino acids, 2g $MgSO_4 \cdot 7H_2O$, pH7.0). Incubation was continued at 37°C for 4-5h, until lysis occurred and the culture allowed to cool overnight at 4°C. RNase A and DNase I were added to a final concentration of 1µg/ml each and incubated at room temperature for 2h. NaCl was added to give a final concentration of 1M prior to incubation on ice for 1h. Bacterial debris was removed by centrifugation at 11,000g for 15 minutes at 4°C. Bacteriophage particles were precipitated in the supernatant by the gradual addition of polyethylene glycol (PEG 8000) to a final concentration of 10% followed by incubation overnight on ice. Precipitated bacteriophage particles were recovered by centrifugation at 11,000 x g at 4°C for 15min, then resuspended in 8ml SM. PEG was removed from the solution by vortexing for 30s with an equal volume of chloroform followed by centrifugation at 3000 x g for 15 min at 4°C. The aqueous phase was removed to a clean FEP tube and EDTA, proteinase K and SDS added to give final concentrations of 20mM, 50 µg/ml and 0.5%(w/v) respectively prior to incubation at 37°C for 1h. Protein and debris were removed by extracting once with phenol equilibrated with 0.1M Tris-HCl (pH 8.0), twice with phenol:chloroform:isoamyl alcohol (25:24:1) equilibrated as for phenol, and once with chloroform:isoamyl alcohol (24:1). Bacteriophage DNA was precipitated from the aqueous phase with 0.1 volume of 3M sodium acetate pH7.0 and 0.7 volumes of isopropanol at room temperature for 30min, and recovered by centrifugation at 14,000 x g for 30min. Precipitated DNA was washed twice with 10ml of 70% ethanol before air drying. The pellet was then resuspended in 500µl of TE (pH 8.0).

5µg of DNA from each lambda clone was digested with *EcoRI*, *SacI* and *SalI* and following electrophoresis and Southern blotting was probed with the *tryI* gene.

2.6.9 Subcloning

Fragments required for subcloning identified by Southern blotting were separated by agarose gel electrophoresis and purified from gel slices using the GeneClean II® Kit (BIO 101 Inc.). Commercially prepared *SalI* digested dephosphorylated pUC18 (Appligene) was used for all ligations from bacteriophage lambda.

Plasmid vector was mixed with a three molar excess of fragment DNA and 1 unit of T4 DNA ligase and 1µl of 10x T4 DNA ligase buffer to give a final reaction volume of 10µl which was incubated at 16°C overnight.

Competent cells, *E.coli* strain JM-109, were prepared by inoculating 100ml of prewarmed LB with a single colony and incubating at 37°C, 200rpm until A_{600nm} of the culture measured 0.4. The culture was chilled on ice for 10 minutes prior to centrifugation at 4000 x g for 10 min at 4°C to harvest the *E.coli*. The pellet was resuspended in 10ml of 0.1M CaCl₂ and stored on ice for 20 minutes. Cells were recovered by centrifugation, and the resultant pellet resuspended in 2ml of ice cold 0.1M CaCl₂ for each 50ml of original culture.

10-50ng of ligated plasmid DNA was added to 200µl of competent cells in a 1.5ml microfuge tube and incubated on ice for 1h. Cells were heat shocked in a water bath at 42°C for 90 seconds then chilled on ice for two minutes prior to the addition of 800µl of LB broth. Cultures were incubated for 1h at 37°C to allow the bacteria to recover. Aliquots of the suspension were spread onto LB agar plates containing 50µg/ml ampicillin. Identification of recombinant plasmids was achieved by α -complementation. Production of active β -galactosidase was detected by spreading 40µl of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal, 20mg/ml in dimethylformamide) and 4µl of isopropylthio- β -D-galactoside (IPTG, 200mg/ml) over the surface of the LB plates which were incubated at 37°C for four hours prior to inoculation with *E.coli*. Plates were inverted and

incubated at 37°C overnight. White colonies were identified as containing recombinant plasmids.

2.6.10 Plasmid DNA preparation

5ml LB containing 50µg/ml ampicillin was inoculated with a single recombinant bacterial colony and incubated overnight at 37°C at 200rpm. 1.5ml of this culture was transferred to a microcentrifuge tube and cells harvested by centrifugation at 12000 x g for 1 minute. Supernatant was decanted and the bacterial pellet resuspended in 100µl of TE pH8.0 and vortexed to a smooth suspension. 200µl of cell lysis solution (1M NaOH, 1% SDS) was added and mixed by gentle inversion. After 1min 150µl of potassium acetate solution (3M potassium acetate, 11.5% glacial acetic acid) was added, the solution mixed five times by inversion followed by incubation on ice for 5min. Precipitated debris was removed by centrifugation at 12,000 x g for 5min and the supernatant transferred to a fresh tube. DNase free RNase was added to a final concentration of 200µg/ml and incubated at 37°C for one hour prior to extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) equilibrated with 0.1M Tris-HCl (pH8.0). DNA was precipitated by the addition of 2 volumes of absolute ethanol for 5minutes at room temperature followed by centrifugation at 12,000 x g for 5min. The DNA pellet was washed once with 70% ice cold ethanol, air dried for 20 minutes and resuspended in 50µl TE (pH8.0).

2.6.11 Sequencing

Plasmid DNA for sequencing was prepared using the Promega Wizard™ Plus Miniprep kit. PCR products were sequenced directly, without prior cloning, following elution of the product from an agarose gel using a Gel-Band purification kit (Pharmacia Biotech) and 100ng sequenced directly using the appropriate primers.

Sequencing was performed with an ABI-377 automated fluorescent sequencer using the Perkin-Elmer dye-terminator kit. Sequence was compiled and analysed by GCG programs (Devereux *et al.*, 1994).

2.6.12 Isolation of full length *SNP1*

This work was performed by Dr. Andy Bailey, at IACR Long Ashton Research Station, Bristol.

Duplicate nylon filters (Hybond-N) representing 15-20,000 plaques from a genomic library of *S.nodorum* isolate LAW were screened, as described previously (Section 2.6.7). The probe for screening on this occasion was the ³²P-labelled 580bp *SNP1* PCR fragment. Hybridisation was at 65°C and final washes were 15min each of 2xSSC/0.1% SDS and 0.5xSSC/0.1%SDS. Eight positives were identified on the first round screen and four were identified on the second round. Following DNA isolation, restriction and Southern blotting, positive bands were subcloned and sequenced. Several different *SalI* fragments were used as templates for sequencing with alternative templates used to confirm sequencing across the *SalI* sites.

Chapter Three: Cell Wall Degrading Enzymes Produced by *Stagonospora nodorum*

3.0 Introduction

Plant pathogenic fungi can secrete a wide range of CWDE during infection and colonisation of host plants (Walton, 1994). The nature of the enzymes produced usually reflects the composition of polymers found in the host cell wall (Cooper *et al.*, 1988). *S.nodorum* grows largely intercellularly during infection of wheat (Bird and Ride, 1981) and can penetrate the host directly (Zinkernagel *et al.*, 1987), implicating CWDE in host colonisation by this fungus. Indeed, production of cell wall degrading enzymes by *S.nodorum* both *in vitro* (Lehtinen, 1993) and *in vivo* (Magro, 1984) has been observed. When grown *in vitro* on wheat cell walls, a range of depolymerases were detected (Lehtinen, 1993). The nine CWDE produced reflected the composition of the wheat cell wall; xylanase activity predominated and only low levels of polygalacturonase were detected. In contrast, during infection of wheat by *S.nodorum*, equal levels of xylanase, polygalacturonase and cellulase activity were observed (Magro, 1984).

Detection of fungal CWDE *in vitro* when grown on host cell walls is useful in establishing the range of CWDE that could be produced by a fungus during host colonisation (Cooper, 1977). It does not however demonstrate a role for any of these enzymes in pathogenicity. To do this, these CWDE ideally need to be detected in infected tissue. Plants are known to produce their own cell wall depolymerases which are probably involved in cell wall turnover (Brett and Waldron, 1996). It is therefore difficult to distinguish plant enzymes from fungal ones by assaying extracts from infected tissue. Despite this it is at least possible to observe differences in enzyme levels between infected and control plants which may implicate a major enzyme in pathogenicity. Subsequently enzyme characteristics such as isoelectric point and molecular weight can be compared in order to reveal their origin.

The aim of this work was to determine which CWDEs are produced by this isolate of *S.nodorum* when grown on wheat cell walls as substrate *in vitro*. Also, as a means of identifying possible key fungal CWDEs *in planta*, differences in enzyme levels between control and infected tissue were studied.

In addition, pre-formed CWDE may sometimes be found associated with spores (Fric and Wolf, 1994) or in the mucilage surrounding spores when they are first produced (Pain *et al.*, 1996). The mucilaginous gel surrounding *S.nodorum* spores produced in pycnidia was assayed for pre-formed enzyme activities. It is possible that these enzymes may remain associated with spores following dilution and rain splash dispersal and are present for initial stages of infection.

3.1 Results

3.1.1 Cell Wall Degrading Enzymes Produced by *S.nodorum* *in vitro*

The aim of this work was to determine which CWDE are induced on wheat cell walls in liquid culture of which some may possibly play a role in pathogenesis. *S.nodorum* was grown in complete medium for 48h to establish a substantial biomass (dry weight $0.4\text{mg} \pm 0.08$). Cultures were then washed with basal salts medium and transferred to basal salts for 12 h. Following starvation, established mycelia were then transferred to 1% wheat cell walls and cultures sampled every 12h for 72 hours.

Polygalacturonase and cellulase activities were low compared to the fast, high production of xylanase which appeared to peak at 24 hours (Fig.3.1). Laminarinase was also high and increased steadily reaching maximum activity at 72h. The Nelson-Somogyi assay for reducing sugars (Nelson, 1944) is not a specific assay for endo-acting glycanases. For example, the xylanase assay will detect any depolymerases showing activity towards the substrate, in the case of birchwood xylan, β -xylosidase, α -glucuronidase and exo-acting xylanases would give “xylanase” activity. An alternative assay for β -1,4 xylanase employs the chromogenic substrate, remazol brilliant blue (RBB) dyed xylan and is reported to be specific for endo β -1,4-xylanase (Biely *et al.*, 1985a). Using this assay maximal xylanase production was between 24 and 48 hours (Fig. 3.3), in contrast to the profile with the Nelson-Somogyi assay. Hence the RBB-xylan assay probably gives a truer picture of actual endo-xylanase activity.

Glycosidase activities were low compared to xylanase and laminarinase but all were detected from 24 hours (Fig.3.2). Glucosidase activity was the highest and it was still increasing at 72h. The second highest activity was arabinosidase, which peaked at 24 hours and remained at this level until 72h. Xylosidase peaked later, at 36h as did acetylsterase. Galactosidase remained fairly low until 60h when it began to increase and was still increasing at 72h.

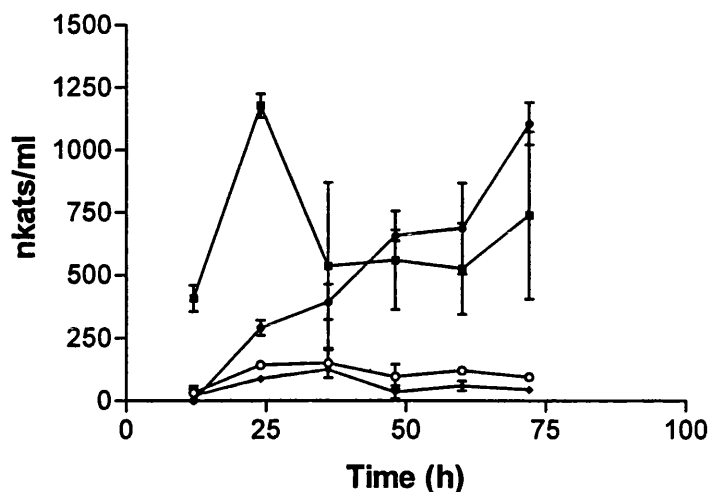


Fig. 3.1. Xylanase (■), laminarinase (•), polygalacturonase (◆) and cellulase (O) produced by *S.nodorum* *in vitro* on 1% wheat cell walls. Activities measured using the Nelson-Somogyi reducing sugar assay. Error bars represent the standard deviation about the mean of three replicate flasks.

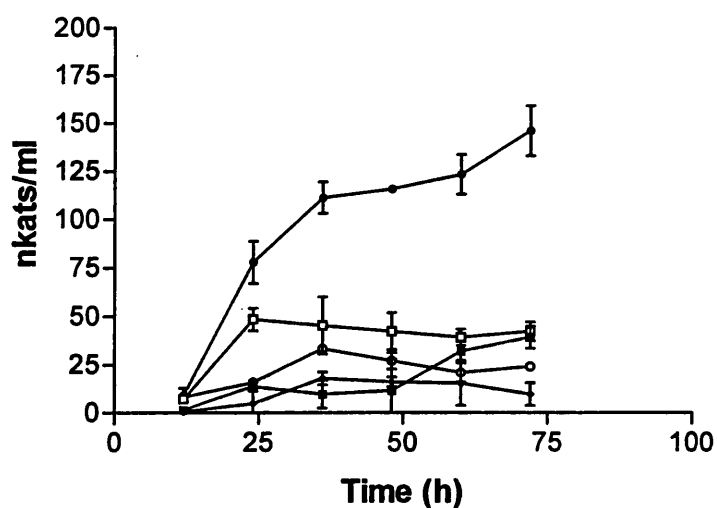


Fig. 3.2. Glucosidase (•), arabinosidase (□), xylosidase (O), acetylesterase (◆) and galactosidase (■) produced by *S.nodorum* *in vitro* on 1% wheat cell walls. Error bars represent the standard deviation about the mean of three replicate flasks.

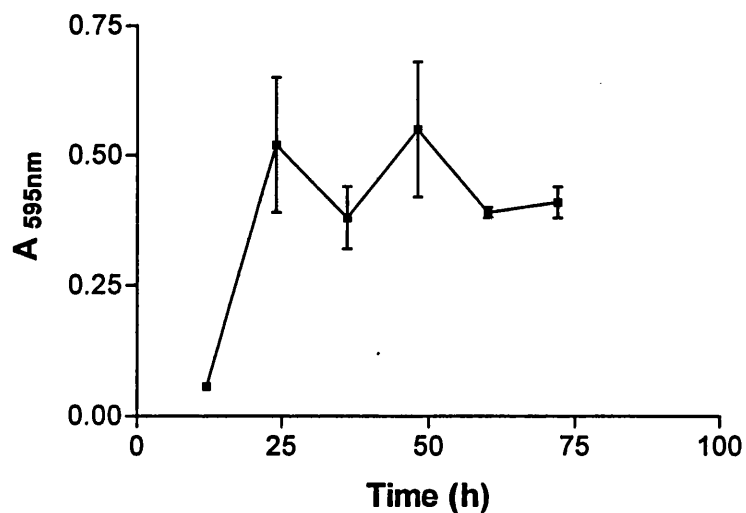


Fig. 3.3. Xylanase produced by *S.nodorum in vitro* on 1% wheat cell walls. Assayed using RBB-dyed-xylan as substrate. Error bars represent the standard deviation about the mean of three replicate flasks.

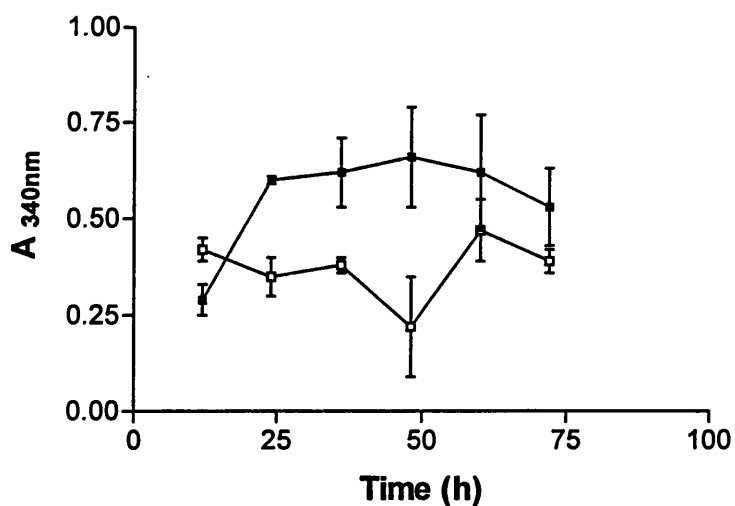


Fig. 3.4. Protease produced by *S.nodorum in vitro* on 1% wheat cell walls. Activity assayed using azocasein at pH 5 (□) and pH 8 (■). Error bars represent the standard deviation about the mean of three replicate flasks.

Protease production also occurred (Fig. 3.4). The major activity detected at pH8, was around twice that measured at pH5. Activity assayed at pH8 was detectable at 12 hours but peaked at 24 hours and remained constant at this level. Activity assayed at pH5 remained fairly constant from 12h.

No pectin lyase or α -glucuronidase activities were detected.

In conclusion, *S.nodorum* produces a range of cell wall degrading enzymes when grown on wheat cell walls, with major activities apparently reflecting the composition of the cell wall. No pectin lyase and very little polygalacturonase enzymes were detected, presumably reflecting the low pectin content of wheat cell walls. Protease activity was also detected, possibly reflecting the importance of protein in the plant cell wall.

3.1.2 Cell wall degrading enzymes produced by *S.nodorum* in planta

The aim of this work was to compare the levels of CWDE in control and infected plants. It was hoped that any clear differences might identify CWDE produced by *S.nodorum* during infection. Seven day-old wheat plants were inoculated with *S.nodorum* and symptoms allowed to develop. Samples were taken at two day intervals over 14 days and enzymes extracted from them. The typical progress of disease symptoms is shown in Table 3.1.

Day	Symptoms on leaves
2	No symptoms
4	Water soaking visible at tips
6	Chlorotic spots appearing
8	Necrotic tip. Necrotic spots surrounded by chlorotic halos
10	Spreading necrotic lesions; extensive chlorosis of the leaf
14	Necrotic split tip, becoming pink as pycnidia form; leaf dead.

Table 3.1. Generalised time course of symptom development of infection of wheat with *S.nodorum* under glasshouse conditions.

Laminarinase showed the highest activity both in infected and control plants (Fig.3.5). Until day six there was slightly more laminarinase in infected than control plants, however, after six days there was more in controls. Xylanase and polygalacturonase activities showed no difference between control and infected plants (Figs 3.6, 3.7), except xylanase at day 14. By day 14, xylanase activity was approximately 6 times greater in infected than controls. No cellulase activity was detected.

Xylanase activity was also assayed using RBB-xylan (Fig. 3.8). Xylanase was first detected 8 days after inoculation, coincident with necrosis, and was still increasing at day 14 as the plants became moribund. No xylanase was detected in control plants. This contrasts with the “xylanase” activity detected in control plants using the Nelson-Somogyi assay (Fig.3.6), probably due to the higher sensitivity and non-specificity of this assay.

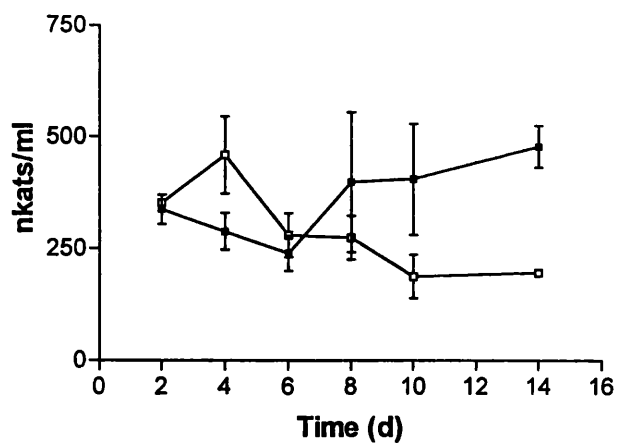


Fig. 3.5. Laminarinase activity in control (■) and infected (□) plants. Error bars represent standard deviation about the mean of three replicate trays.

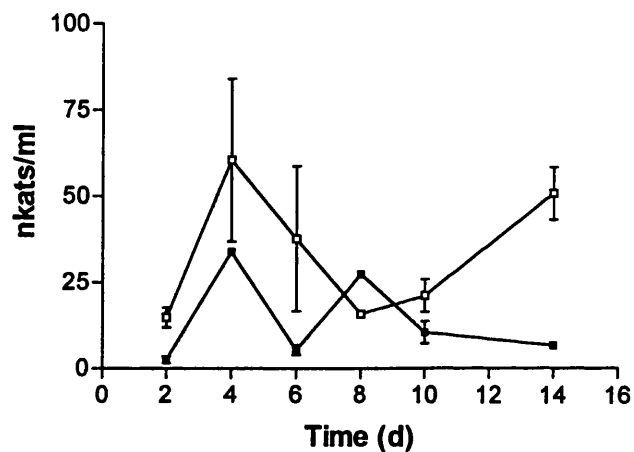


Fig. 3.6. Xylanase activity in control (■) and infected (□) plants as detected with the Nelson-Somogyi reducing sugar assay. Error bars represent the standard deviation about the mean of three replicate trays.

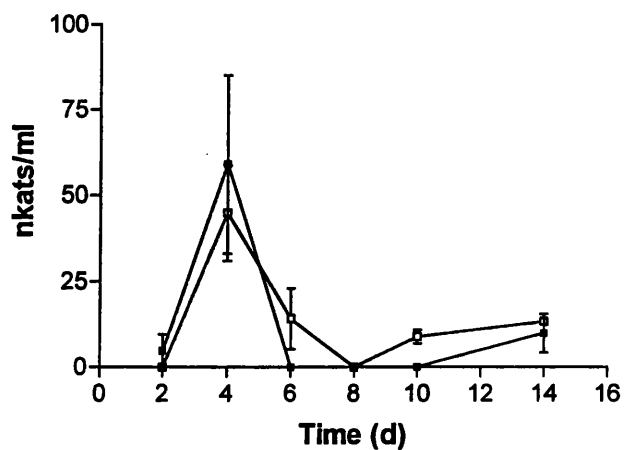


Fig. 3.7. Polygalacturonase activity in control (■) and infected (□) plants. Error bars represent the mean of three replicate trays.

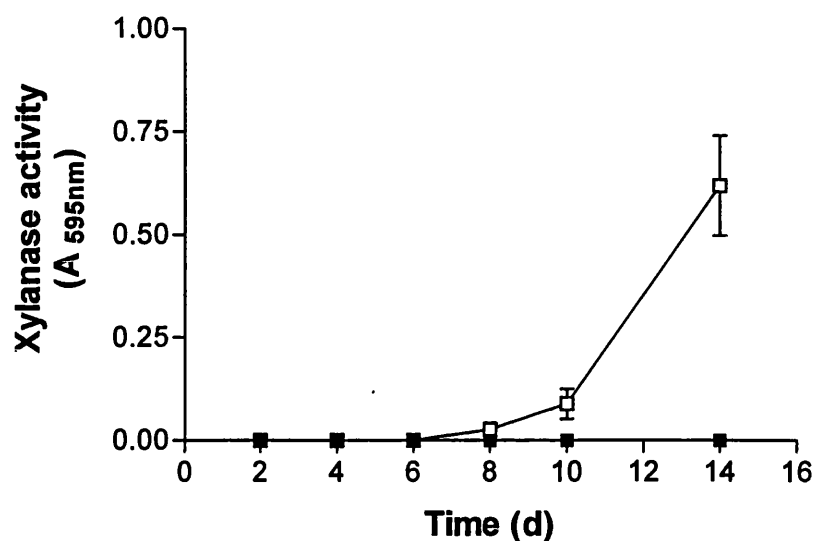


Fig. 3.8. Xylanase activity in control (■) and infected (□) plants measured with RBB-dyed-xylan. Error bars represent the standard deviation about the mean of three replicate trays.

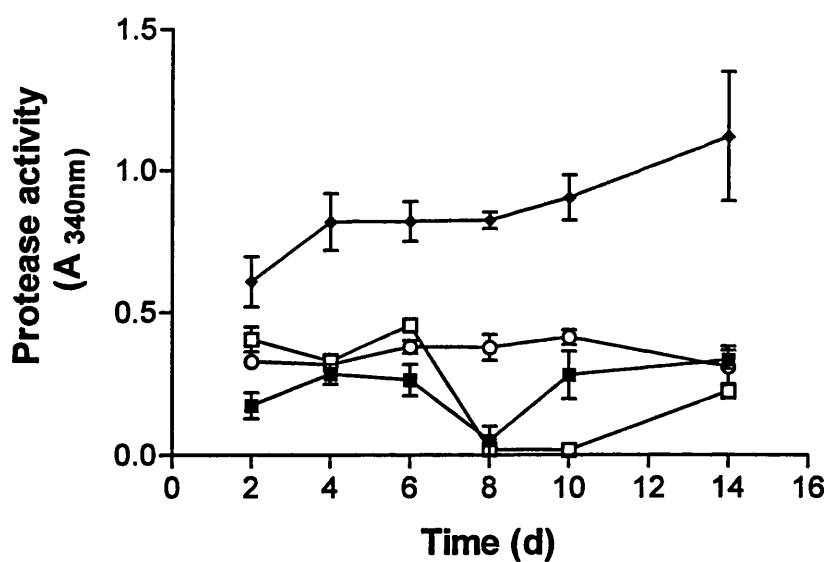


Fig. 3.9. Protease activity assayed at pH5 in control (O) and infected plants (◆) and at pH 8 in control (■) and infected (□) plants. Error bars represent the standard deviation about the mean of three replicate trays.

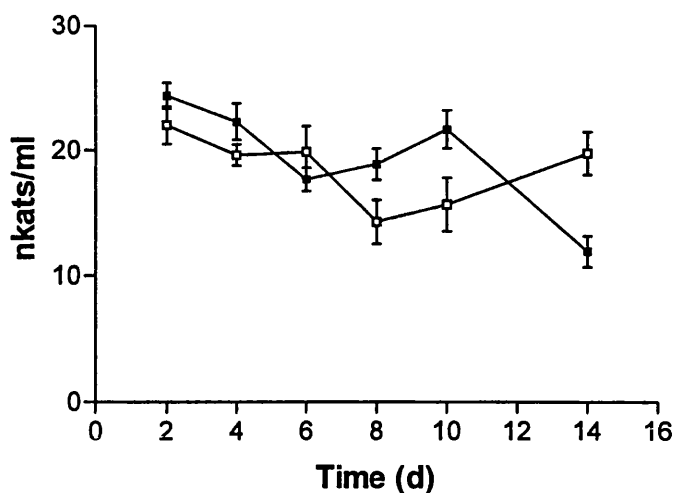


Fig. 3.10. Galactosidase activity in control (■) and infected (□) plants. Error bars represent standard deviation about the mean of three replicate trays.

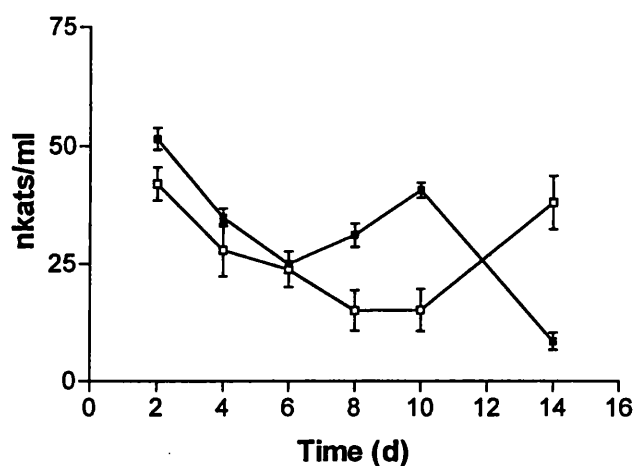


Fig. 3.11. Glucosidase activity detected in control (■) and infected (□) plants. Error bars represent the standard deviation about the mean of three replicate trays.

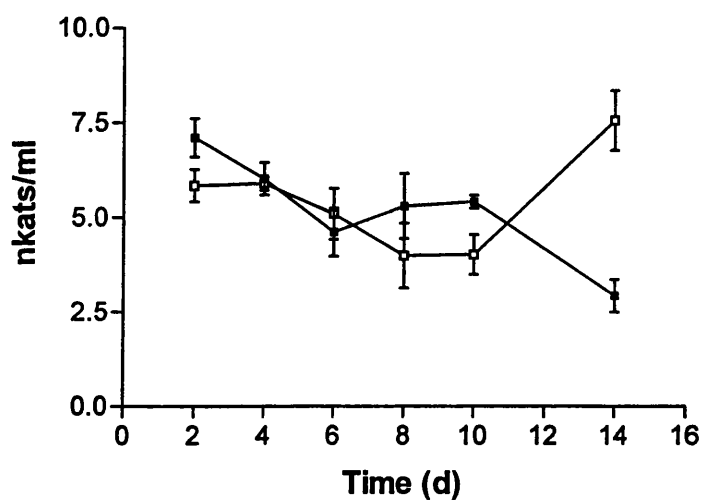


Fig. 3.12. Xylosidase activity in control (■) and infected (□) plants. Error bars represent standard deviation about the mean of three replicate trays.

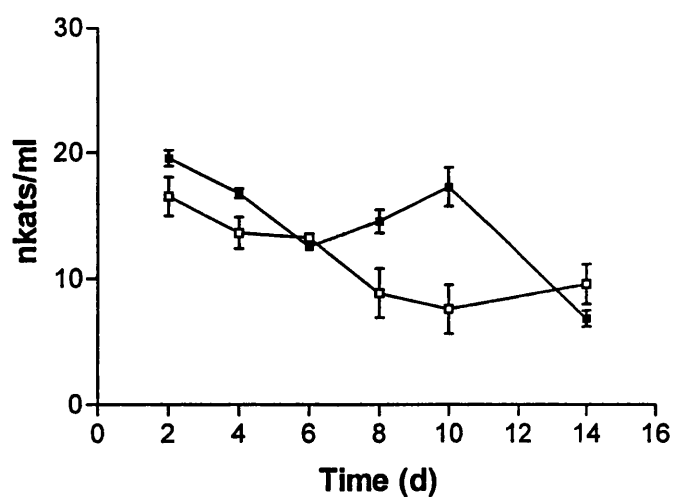


Fig. 3.13. Arabinosidase activity in control (■) and infected (□) plants. Error bars represent the standard deviation about the mean of three replicate trays.

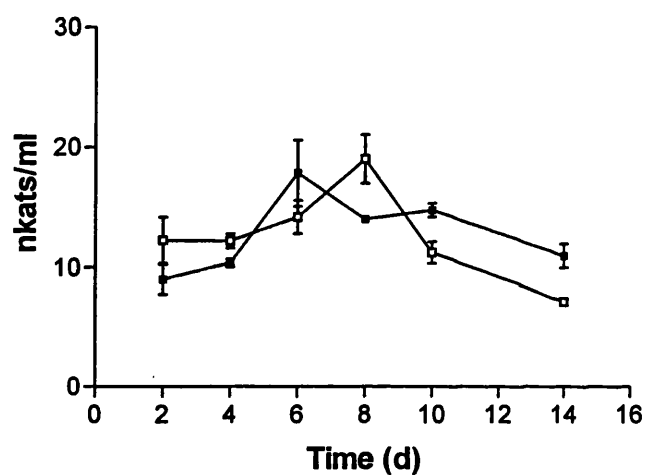


Fig.3.14. Acetylcholinesterase activity in control (■) and infected (□) plants. Error bars represent standard deviation about the mean of three replicate trays.

Protease activity (pH5) showed higher levels of activity in infected tissue than in control plants from day 2 (Fig. 3.9). This continued to rise to up to four times that of control plants by day 14. Protease activity (pH8) was higher in infected than control plants until day 8. After day 8, pH8 protease activity was higher in control plants, although protease activity in infected plants began to rise again at day 14.

Glycosidase activities (Figs.3.10-3.14) were much lower than xylanase and laminarinase, as was observed *in vitro*. Similarly, β -glucosidase and arabinosidase activities were produced at the highest levels, along with acetylsterase. There were no significant differences observed between control and infected plants for any of the glycosidases. Again, no α -glucuronidase or pectin lyase activity was detected.

It is clear from these studies that it is impossible to separate host and pathogen enzyme activities purely by assaying plant extracts. This study therefore only gives an indication of which enzymes may be involved in pathogenicity. Another problem concerns the comparison of enzyme levels in healthy and moribund plants. For example, at day 14 there may be little difference in enzyme activity between infected and control plants, but the plant cells in infected tissue are mainly dead, so the enzymes from this material are likely to be of pathogen origin. The range of enzymes produced *in planta* mirror those produced *in vitro* on wheat cell walls, confirming their use as a relevant source of inducers for fungal wall depolymerases.

The most significant enzyme activities detected in these studies, both *in vitro* and *in planta* were laminarinase, xylanase and protease. Laminarinase however is produced at equally high levels by the host plant and this therefore eliminates it as a potential fungicide target for disease control. Xylanase however, was produced at significantly higher levels in infected plants later in infection, and is likely to be of fungal origin. Protease was produced at high levels in infected tissues compared to controls throughout infection, although its origin is unclear.

Such high levels compared to controls warrant further investigation. The conclusion from this initial work was to characterise the xylanase and protease activities further as enzymes potentially important in pathogenicity.

3.1.3 Detection of CWDE in cirrus mucilage

It was the aim of this work to assay cirrus mucilage surrounding spores of *S.nodorum* for the presence of CWDE. Because of the difficulties in obtaining large numbers of pycnidia and hence enough mucilage from infected tissue an *in vitro* system was used instead. Cirrus mucilage is readily produced from pycnidia formed on agar plates under the standard culture conditions for *S.nodorum*. To maximise production, large (27cmx27cm) petri dishes were used to generate a large amount of cirrus mucilage (Fig. 3.15).



Fig. 3.15. Growth of *S.nodorum* on large petri dishes. Note the cirrus mucilage produced. Magnification (x 0.66)

Mucilage was removed from the plates by pipetting and spores removed by centrifugation at 11,000 g for 15min, or until all spores were removed. Removal of spores was confirmed by microscopy. Numbers of spores were typically around $4 \times 10^7 \text{ ml}^{-1}$ of mucilage.

Enzyme	Assay	Activity (SD)
Laminarinase	Nelson-Somogyi	13490 nkats/ml (950)
Xylanase	Nelson-Somogyi	2850 nkats/ml (350)
Xylanase	RBB-xylan	Abs. 595nm 0.51 (0.03)
Xylosidase	<i>p</i> -nitrophenol-linked (PNP)	61.5 nkats/ml (0.03)
Arabinosidase	PNP	50.25 nkats/ml (5.75)
Acetylesterase	PNP	43.9 nkats/ml (1.9)
β -glucosidase	PNP	71.3 nkats/ml (1.9)
Esterase	PNP	70.35 nkats/ml (0.95)
Galactosidase	PNP	35.4 nkats/ml (2.1)
Protease pH5	Azocasein	Abs 340nm 0.365 (0.025)
Protease pH8	Azocasein	Abs 340nm 0.995 (0.105)

Table 3.2. Enzyme activities detected in cirrus mucilage

Cirrus mucilage was assayed for the presence of the CWDE that had previously been detected *in vitro* on wheat cell walls. All of the same enzyme activities were present (Table 3.2). As found *in vitro* on wheat cell walls the activity of the glycosidases was low compared to laminarinase and xylanase. Once again β -glucosidase was the predominant glycosidase detected. In contrast to the liquid cultures, laminarinase activity was almost five times greater than the xylanase activity and was the highest activity detected. Protease activity (pH8) was three times greater than that detected at pH5, similar to the levels detected in liquid cultures. An additional activity detected, not assayed previously, was non-specific esterase, assayed against the general esterase substrate *p*-nitrophenyl butyrate.

The protein (Bradford, 1976) and carbohydrate (Dubois *et al.*, 1956) concentration of the mucilage was determined and found to be 8 and 72.3 mg/ml respectively.

3.2 Discussion

3.2.1 CWDE produced *in vitro*

S.nodorum produced a wide range of CWDE when grown *in vitro* on wheat cell walls as sole source of carbon. The range of enzymes detected reflected the composition of the host cell wall. One of the first enzymes to be produced and in the largest amounts was β -1,4 xylanase. Cereal cell walls contain high amounts of β -1,4-xylans and hence xylanase production is thought to be important for pathogens of cereals in achieving penetration (Peltonen, 1995). This early and high production of xylanase by *S.nodorum* on wheat cell walls was also observed in previous studies by Lehtinen (1993) and Magro (1984). A similar pattern of xylanase production has been observed for other cereal pathogens *in vitro* on wheat cell walls. These include, *Rhizoctonia solani*, *Fusarium culmorum* and *Pseudocercospora herpotrichoides* (Cooper *et al.*, 1988). Detection of xylanase production early in infection of cereals contrasts with their late production by pathogens of dicotyledons (Cooper *et al.*, 1988). When *R.solani* was grown on potato cell walls instead of wheat much lower, later production of xylanase was observed (Cooper *et al.*, 1988). This may reflect the accessibility of xylan, found abundantly in the primary wall of monocots but only in low amounts in the secondary wall of dicots. Correspondingly, xylanases have been shown to be more effective at degrading monocotyledon cell walls compared to those of dicotyledons (Baker *et al.*, 1977). It is also possible that xylanases may be important during saprophytic growth of fungi on cereals (Flannigan and Sellars, 1978). Xylose serves as a good carbon source for many fungi, often superior to glucose (Flannigan and Sellars, 1978).

The second most abundant enzyme was laminarinase (β -1,3 glucanase). Such high levels of this enzyme were not observed in a similar study by Lehtinen (1993), although some activity was detected. In her study, spores were inoculated directly into cultures of wheat cell walls so results are difficult to compare to the established-biomass transfer method used in this work, which

generates a rapid burst of enzyme production. Laminarinase production is expected due to presence of mixed (β -1,3- and β -1,4-) linked glucans in the monocotyledon cell wall. Indeed its production *in vitro* has been observed for a number of other cereal pathogens (Cooper *et al.*, 1988), suggesting that laminarinase may be capable of wall degradation and may play a role in penetration. Laminarinase may also play a role in overcoming callose deposition by the host. Callose, a β -1,3 glucan found in papillae at sites of fungal infection, has been shown to be degraded by the purified β -1,3-glucanase secreted by the maize pathogen *Cochliobolus carbonum* (Van Hoof *et al.*, 1991). It is interesting to note that this enzyme was unable to degrade oat mixed-linked glucans, suggesting that it would be incapable of degrading the host wall but could assist in penetration by degrading callose. β -1,3 glucanases are also thought to play a role in fungal differentiation and autolysis given that the fungal cell wall comprises β -1,3 glucan; however these glucanase forms tend to be wall bound (Bielecki and Galas, 1991). Secretion of these potentially self-destructive enzymes by fungi seems erroneous, particularly as they are also produced by plants in response to fungal attack (Mauch *et al.*, 1988). This paradox may have been resolved by the discovery that the laminarinase secreted by *C. carbonum* is exo-acting. This type of action causes less disruption to wall integrity than an endo-acting enzyme, particularly in the fungal cell wall where the glucan is heavily branched through β 1,6 linkages which makes them even less susceptible to exo-acting laminarinases. Plant β -1,3 glucanases are known to be largely endo-acting and so will be more destructive to the fungus.

A wide range of glycosidase activities, involved in xylan and glucan degradation were detected *in vitro* as also previously reported by Lehtinen (1993). These glycosidases were produced at significantly lower levels than the xylanase and laminarinase activities detected. This may reflect the low numbers of these types of linkages in the wall compared to those in the main backbones, cleaved by endo-xylanase and endo-laminarinase. Although they may be produced at lower levels, their activity may be very important in removing side groups, relieving steric hindrance and allowing other endo-enzymes to act. Xylanases frequently

do not cleave linkages adjacent to units carrying a side chain (Biely, 1985). In fact the pure xylanase of *Trichoderma pseudokonigii* only hydrolysed approximately 15% of the glycosyl linkages in glucuronoarabinoxylan (Darvill *et al.*, 1980). Arabinosidases facilitate glucuronoarabinoxylan hydrolysis by cleaving arabinose side chains and glucuronidases remove glucuronic acid side chains. It was unusual in this study that no α -glucuronidase activity was detected given the presence of glucuronic acid side chains on xylan (Carpita, 1996). The peak in arabinosidase activity coincided with the peak in xylanase activity, consistent with the synergistic action of these enzymes. A similar pattern of expression of arabinosidase activity on wheat cell walls was observed in *R.cerealis* (Cooper, *et al.*, 1988). The virulence of chemically induced mutant strains of *Sclerotinia fructigena* on apple has been correlated with α -arabinosidase producing ability rather than with polygalacturonase or pectin methyl esterase (Howell, 1975). β -Xylosidase activity peaked later, possibly cleaving xylooligosaccharides produced by the action of endo-xylanase, to xylose. Also acetylsterase reached highest levels at this time and it is also thought to be important in synergistic degradation of xylan. Arabinoxylan is often acetylated and deacetylation has been shown to increase its susceptibility to degradation by xylanases (Wood and McCrae, 1986b). The low galactosidase activity reflects the low galactose content of the wheat cell wall, although production increased in later cultures. The glycosidase produced in the greatest amount was glucosidase. Glucosidases are known to act together with endo β 1-4 glucanases and cellobiohydrolases in degrading cellulose. However, few studies have looked at their involvement in the degradation of other β -glucans. Synergy between β -glucosidase and β 1-3 glucanase has been demonstrated in *Acremonium pericinum*, enhancing glucan degradation by 6 fold (Piston *et al.*, 1997). This suggests a role for β -glucosidase in assimilation of β -glucans as carbon sources through hydrolysis of oligoglucosides released by β 1-3 glucanase action. The timing of production of β -glucosidase by *S.nodorum* followed the same pattern as laminarinase suggesting such a synergy could exist here. It is also possible that β -glucosidase is capable of acting alone; it has been correlated

with the pathogenicity of *Botrytis cinerea* and the pure enzyme is capable of degrading apple cell walls (Sasaki and Nagayama, 1996).

Production of polygalacturonase was low and no pectin lyase activity was detected, reflecting the low levels of pectin found in the wheat cell walls. This is in contrast however to Lehtinen (1993) who detected higher levels of polygalacturonase than laminarinase and also Magro (1984) who detected higher levels of polygalacturonase than xylanase when *S.nodorum* was grown on wheat cell walls. Both of these studies used cultures where spores were inoculated directly; the resulting long, slow growth of cultures may account for some differences in levels of enzymes produced. Low levels of PG and PL were detected when *R.cerealis* and *F.culmorum* were grown on wheat cell walls, with virtually undetectable levels produced by *P.herpotrichoides* (Cooper *et al.*, 1988). In this same study *R.cerealis* was also grown on potato cell walls and β -galactosidase and pectin lyase activity increased compared to activity on wheat cell walls. Correspondingly, xylanase activity decreased due to the fungus adapting its CWDE to the change in host cell wall structure. Pectin degrading enzymes are required by many pathogens of dicotyledons (Cooper, 1984) because pectin constitutes a major component of the dicotyledon cell wall. They are the first CWDE produced *in vitro* when pathogens such as *Verticillium albo-atrum* and *Colletotrichum lindemuthianum* are grown on host cell walls (Cooper, 1977). The ability of these enzymes to macerate the host cell wall has been clearly demonstrated (Bateman and Basham, 1976)

Cellulase levels in this study were low, as observed previously (Lehtinen, 1993; Magro 1984). Cellulase is expected to be observed later in cell wall degradation as cellulose becomes accessible to enzymic attack (Ward and Moo-Young, 1989) and isn't generally considered an important enzyme in pathogenicity. However, in a few cases it is active during early stages of infection and the pathogenicity of isolates of *P.solanacearum* clearly correlates with cellulase production (Kelman and Cowling, 1965).

Also detected *in vitro* was protease activity; this has been previously found during *S.nodorum* infection of seeds (Sieber, 1989) but was not detected by Lehtinen (1993). The assay used by Lehtinen (1993) was based on the degradation of BSA and detection of amino acids using the Folin reagent which is known to be unreliable (Dunn, 1989a). Lehtinen (1993) did however detect protease activity when *S.nodorum* was grown on skimmed milk agar plates (also observed in this work, results not shown). Proteases are produced by many fungi when grown on host cell walls including *C.carbonum*, *V.dahliae*, *Fusarium* spp., *Ustilago maydis*, *Ophiostoma piceae* and *C.lindemuthianum* (Murphy and Walton, 1996; Lambert and Pujarniscle, 1984; Urbanek and Yirdaw, 1978; Hellmich and Schauz, 1988; Abraham and Breuil, 1995; Ries and Albersheim, 1973). They have however been largely overlooked in studies of CWDE, which have tended to focus on polysaccharide degrading enzymes. This may be a result of the extensive glycosylation of wall proteins that makes them innately resistant to degradation (Lamport, 1980). Proteins can comprise 10% of the plant cell wall (Showalter, 1993) and can increase in response to challenge by pathogens (Mazau and Esquerre-Tugaye, 1986). Hence proteases may serve as CWDE, although to date only three proteases from plant pathogens have been found that are capable of degrading cell wall proteins (Heilbronn *et al.*, 1995; Willis *et al.*, 1987; Dow *et al.*, 1998). The most recently characterised, a metalloprotease from *Xanthomonas campestris* specifically degrades proline/hydroxyproline rich plant cell wall glycoproteins (Dow *et al.*, 1998). Alternatively proteases may be involved in protection from antifungal plant proteins such as chitinases and glucanases (Mauch *et al.*, 1988). Finally they may play a role in enzyme (Moormann *et al.*, 1993) or toxin (Howard and Buckley, 1985) processing. The production of protease inhibitors by plants in response to infection suggests that they may be important enzymes in pathogenesis (Ryan, 1990).

Generally, CWDE production is sequential in the order of accessibility of polymers to enzyme attack. For pathogens of dicots the sequence is pectinases, hemicellulases and finally, cellulases (Cooper, 1977). The hemibiotrophic fungus *C.lindemuthianum* produces a sequence of cell wall degrading enzymes *in*

vitro on bean cell walls of PG, PL and protease followed by neutral polymer degrading enzymes of arabinosidase and galactosidase (Wijesundera *et al.*, 1989). Cereal pathogens in contrast, as seen here, only produce low levels of pectinases while an early, high production of xylanases is followed by other hemicellulases, proteases and then later, sometimes cellulases. More work is required to determine the regulation of the CWDE of *S.nodorum*. Many CWDE are substrate induced and catabolite repressed although some are constitutively expressed (Annis and Goodwin, 1997). It is not possible from this study to know whether the enzymes are induced and/or derepressed in the presence of wheat cell walls; other critical studies using a range of carbon sources are required to determine this.

3.2.2 Cell wall degrading enzymes produced *in vivo*

Generally, less work has been done on cell wall degrading enzymes *in planta* because of the difficulties in distinguishing host and pathogen enzyme activities. Magro (1984) found xylanase to be the predominant activity produced by *S.nodorum* in wheat plants compared to polygalacturonase and cellulase, with only low levels of activity in control plants. This has been confirmed by this work, although higher levels of xylanase in infected plants were only detected in the later stages of infection. In contrast, xylanase synthesis occurred early during infection of wheat seedlings by *R.cerealis* (Cooper *et al.*, 1988) and by *P.herpotrichoides* (Mbwaga *et al.*, 1997). The “xylanase” activity detected in control plants by the Nelson-Somogyi assay is consistent with other studies which have detected the presence of xylanase in uninfected wheat plants (Cooper *et al.*, 1988; Mbwaga, *et al.*, 1997).

Laminarinase production was high in both control and infected plants with no significant differences between them. This was also found in infection of wheat seedlings by *R.cerealis* (Cooper *et al.*, 1988). Very similar levels of glycosidase activities were detected in both control and *S.nodorum* infected plants in this work and this was the same for infection of wheat seedlings by *R.cerealis*, with

the exception of arabinosidase and galactosidase which increased significantly, later in infection (Cooper *et al.*, 1988). No increase in polygalacturonase activity was detected in infected plants compared to controls and no pectin lyase activity was detected at all. This contrasts with the work of Cooper *et al.* (1988) on *R.cerealis* in which high levels of PG and PL were detected in older lesions. Several pectic enzymes were also detected at elevated levels compared to controls after infection by *P.herpotrichoides* (Mbwaga *et al.*, 1997). This activity, as with *R.cerealis* was greatest at advanced stages of disease. This late production or absence of pectic enzymes reflects the low amount of pectin in monocot cell walls and contrasts with dicot pathogens where pectic enzymes are produced early in infection e.g. in cavity spot of carrots caused by *Pythium ultimum* (Campion *et al.*, 1997). This was not the case however during infection of wheat roots by *G.graminis* where PG activity was detected prior to the appearance of symptoms (Dori *et al.*, 1992). The very low levels of PG detected in this study on *S.nodorum*, may be due to the presence of a PG inhibitor, found in the walls of many plants (Albersheim and Anderson, 1971). For example in *Monilinia* infected fruits, PG activity was only detected after removal of inhibitors(s) by isoelectric focusing (Fielding, 1981). Another PG inhibitor was detected in extracts of bean, infected with *C.lindemuthianum* (Wijesundera *et al.*, 1989). However, *S.nodorum* only produced low levels of PG *in vitro* on wheat cell walls, so the low activity detected during infection is probably real.

Cellulase activity was not detected in either control or *S.nodorum* infected wheat plants. A lack of cellulase activity was also observed in wheat infected by *R.cerealis* (Cooper *et al.*, 1988). It is generally thought that cellulases would be produced later in infection as cellulose becomes accessible to attack, as is observed *in vitro*. This was the case during infection of wheat by *P.herpotrichoides* where cellulase peaked four weeks after inoculation (Mbwaga *et al.*, 1997). This contrasts with wheat roots infected by *G.graminis* where cellulase activity was detected at the same time as the appearance of visual symptoms (Dori *et al.*, 1995). Interestingly, disease developed more slowly when the inoculum was previously grown on a medium containing glucose rather than

carboxymethyl cellulose (CMC). These concentrations of glucose did not repress cellulase activity, rather CMC induced production of cellulolytic enzymes, and enhanced the ability of the pathogen to colonise the roots.

Protease activity was detected at elevated levels in infected tissue compared to controls, particularly when assayed at pH5. Aspartic protease activity has been detected in carrots infected with *Botrytis cinerea* (Movahedi and Heale, 1990). No activity was found in control plants in their study and the activity in infected tissue showed many similarities to the purified aspartic protease secreted by *B.cinerea* in culture. Another protease of fungal origin was detected in bean infected with *C.lindemuthianum* (Wijesundera *et al.*, 1989), although other proteases of host origin were also present. Acid protease activity was also detected during infection of maize seedlings by *F.culmorum*, *F.avenaceum* and *F.oxysporum* (Urbanek and Yirdaw, 1978) with only very low levels detected in control plants. At this stage it is impossible to say whether this protease activity detected *in planta*, or that of any of the other enzymes detected, is produced by *S.nodorum* or the host plant, wheat. Plants are known to be able to produce all the enzymes detected in this study (Brett and Waldron, 1996) and so more detailed investigations are required to distinguish fungal and plant activities. This has been achieved in some systems by comparing the isoforms of CWDE produced *in vitro* by the fungus, with those found in infected and healthy tissue. For example, *C.lindemuthianum* when grown in liquid culture on bean cell walls secretes two isoforms of pectin lyase and protease, and one isoform each of arabinosidase, α -galactosidase, β -galactosidase and polygalacturonase. Following determination of the pI and molecular weight of these isoforms, isoelectric focusing was performed on infected and control bean extracts. All three fungal glycosidases were found in infected tissue but only one form of the pectin lyase and only one form of the protease were found, based on isoelectric point and molecular weight. At first the second isoform of protease also appeared to be present in infected tissue, but a protease with the same isoelectric point was also present in control tissue. The molecular weight of this protease differed from the one produced *in vitro* by the fungus indicating that this protease

was probably of host origin (Wijesundera *et al*, 1989). As this study showed, production of isoforms of enzymes *in vitro* does not necessarily mean that they will be produced *in vivo*. Also, it is possible that other isoforms will be produced by the fungus *in vivo* that were not detected *in vitro*. This was found during infection of carrot roots by *Mycocentrospora acerina* where two new isoforms of pectin methylesterase were detected, not previously seen in culture (LeCam *et al*, 1997). In these cases it is difficult to evaluate whether the new isoforms appearing in infected tissue are fungal or induced in the plant in response to infection. The drawback of studying CWDE *in vitro* and then focusing on the wrong isoforms can be minimised by using host cell walls as the inducing substrate. While this does not eliminate the problem it should induce the majority of enzymes and is far better than using commercial substrates such as xylan or pectin to induce enzyme production. This was clearly seen with *Erwinia chrysanthemi* which produces two sets of separately inducible pectate lyase isoforms. One set is induced on pectate, plant cell walls and *in planta*, while the other, known as the 'plant-inducible' set are only produced *in planta* or on plant cell walls *in vitro* and are not induced by pectate (Kelemu and Collmer, 1993).

In this study on *S.nodorum*, the range of CWDE produced *in vitro* on wheat cell walls was similar to those detected in infected plants, although their origin in infected plants is uncertain. That *S.nodorum* can produce this whole range of enzymes has at least been demonstrated. This circumstantial evidence implicates a possible role for xylanase and protease in pathogenesis.

3.2.3 Cell wall-degrading enzymes in cirrus mucilage

S.nodorum produces a mucilage at the time of sporulation that surrounds the pycnidiospores. Previous studies on this mucilage have reported that it contains a germination inhibitor to prevent the spores from germinating within the pycnidia. The composition of the mucilage is 60% spores and 40% proteins and carbohydrate (Fournet, 1969). This study detected a similarly high protein and carbohydrate content, with protein levels comparable with that observed for

mucilage from *C.graminicola* but with a considerably higher carbohydrate content (Ramadoss *et al.*, 1985). Following dilution of the cirrus extract by rain splash it is then possible for spores to germinate and dilute extracts of mucilage have been shown to stimulate germination of pycnidiospores (Rapilly and Skajenikoff, 1974). It is possible that the high protein and carbohydrate content of the mucilage provides nutrients to support the extensive epiphytic growth of *S.nodorum* on the leaf surface prior to penetration (Baker and Smith, 1978).

Extracellular mucilages are common amongst fungal plant pathogens and can be produced by fungal spores and infection structures. They may play a role in adhesion of spores and infection structures (Nicholson and Epstein, 1991), they may provide a protective environment (Moloshok *et al.*, 1993) or they may provide a medium for the deployment of fungal enzymes during infection (Deising *et al.*, 1992). Conidia of many fungi, including a number of important plant pathogens are produced within a copious mucilaginous matrix (Lewis and Cooke, 1985a). The protective properties of the mucilage have been studied in a number of cases. In *Leptosphaeria maculans*, *Mycosphaerella pinodes* and *Septoria apiicola* this mucilage provided protection for spores from extremes of temperatures, changes in relative humidity and U.V radiation (Lewis and Cooke, 1985a). In *Colletotrichum graminicola* it also serves as an antidesiccant (Nicholson and Morares, 1980). This particular spore mucilage is probably the best characterised, the principal component of which is a high-molecular-weight complex of glycoproteins composed of mannose, rhamnose, galactose and glucose (66,22,10 and 2 mole% respectively) (Nicholson and Epstein, 1991). These glycoproteins, particularly proline-rich proteins, have an exceptionally high affinity for binding phenolic compounds and are known to protect the spores of *C.graminicola* from the toxic phenols produced by plants in response to infection (Nicholson *et al.*, 1986). The high carbohydrate and protein content of the mucilage of *S.nodorum* detected in this study may provide similar protection. Further studies are required to determine whether the carbohydrate detected is present as glycoprotein rather than as polysaccharide and also what types of amino acids are present. In another study on *C.graminicola* the amino acid

composition of the conidial mucilage was found to be 50% hydrophobic amino acids, 23% hydroxylic (threonine and serine) and very low levels of aromatic amino acids. These types of amino acids and the unusually high concentrations of proline (11mole%) (known to bind phenols) are also characteristic of animal mucins (Ramadoss *et al.*, 1985).

Enzyme activity has been frequently associated with these conidial mucilages but there have been no previous reports of enzyme activity associated with the cirrus mucilage of *S.nodorum*. During this study a wide range of CWDE were detected, some of remarkably high activity. One of the activities detected was a serine esterase, possibly a cutinase, detected with the substrate *p*-nitrophenyl butyrate. It is likely that this activity is a cutinase in view of previous detection of a cutinase in ungerminated pycnidiospores (Dewey *et al.*, 1997). The cutinase in spores was inhibited by the specific cutinase inhibitor diisopropylfluorophosphate (DFP) and on a western blot reacted with an anti-cutinase monoclonal antibody to give a band at approximately 45kDa. The presence of arabinosidase, glucosidase, galactosidase and protease activity is also consistent with the work of Dewey *et al.* (1997) who detected these activities in washed, ungerminated spores. The presence of acidic protease is consistent with their detection of a protease with a pH optimum of 4.0. However, the alkaline protease was not detected in washed spores, suggesting that this may only be found in the mucilage. Also, the presence of xylanase and xylosidase activities in the mucilage contrasts with their absence in washed ungerminated pycnidiospores, again these may only be present in mucilage. Additional activities detected in the mucilage and not assayed for previously by Dewey *et al.* (1997) were acetylsterase and laminarinase. Further work is required to determine what other enzyme activities are present in the mucilage of *S.nodorum*.

Enzyme activities have been found associated with the conidial matrix of a number of plant pathogens, of which the most intensively studied of these are *Colletotrichum* spp. The conidial matrix of *C.graminicola* contains invertase, β -glucosidase, non-specific esterase, cutinase, DNase, RNase, laccase, protease and

alkaline phosphatase (Nicholson and Epstein, 1991). Similarly, the conidial matrices of both *C.gloesporioides* and *Mycosphaerella pinodes* contain invertase, cellulase, PL and PG (Lewis and Cooke, 1985b). The exact role of these enzymes in disease development is unknown. Laccase, a polyphenol oxidase, may protect spores by eliminating toxic plant phenols from the immediate environment of germinating conidia (Anderson and Nicholson, 1996). One crucial factor in determining the role of these enzymes will be discovering whether they remain associated with spores following dispersal. There is some evidence that the enzyme activities present in the conidial mucilage of *C.graminicola* remain associated with the spores after dispersal (Pascholati *et al.*, 1993). Following dilution of *C.graminicola* spores, cutinase activity remained in a zone immediately surrounding conidia as detected by hydrolysis of inoxyzacetate. If these enzymes remain associated with spores after dispersal they may play an important role in the early stages of disease. For example, the cutinase may release cutin monomers from the leaf that may induce cutinase production by the spore for penetration. These enzymes may also release essential nutrients to spores during the pre-penetration phase and their localised action may also facilitate a more rapid penetration when spores germinate. The conidial matrix of *C.orbiculare* contains pectinesterase, cellulase, invertase and esterase activity. The addition of this matrix to spores advanced disease development by 1 day (McRae and Stevens, 1990). Also, the medium that the spores were produced on affected the range of enzymes produced in the matrix and this also affected disease symptoms. Despite the evidence that the enzymic activity remains associated with spores it is unclear whether the mucilage remains associated as well, and if so what its role is following spore dispersal. One possibility is adhesion, although the conidial mucilage of *C.graminicola* has been shown not to be involved in adhesion of spores (Mercure *et al.*, 1994).

It would be interesting to compare the range of enzymes in the mucilage of pycnidia produced by *S.nodorum* on the leaf surface to those produced *in vitro* to confirm that the spectrum is the same. This could be done by assay combined with isoelectric focusing to determine which isoforms of the enzymes are present.

It is interesting that this wide range of CWDE are present in the conidial mucilage produced on a synthetic medium. This suggests that their production is developmentally regulated as no substrates or inducers for many of these enzymes, e.g. xylanase, are present in the medium. This supports their possible role in infection of the plant as it appears that they are generated as 'pre-packaged' enzymes which will be available to the fungus from the moment it lands on the leaf. Their presence may be required for spore adhesion or host penetration. Developmental regulation of CWDE production has been demonstrated for other fungi at different stages of infection in the absence of enzyme substrate. For example, *Uromyces viciae-fabae* produces a range of differentiation specific CWDE (Deising *et al.*, 1995). It has been possible to study these because *U. viciae-fabae* can be induced to differentiate infection structures on artificial substrata. Enzymes produced in this way include, cellulase, protease and pectin methylesterase.

The presence of both xylanase and protease activities at high levels in the cirrus mucilage supported their selection for further characterisation.

Chapter Four: Proteases of *S.nodorum*

4.0. Introduction

Proteolytic enzymes are degradative enzymes which are capable of cleaving peptide bonds in proteins to generate peptides and amino acids. Historically their function was considered as purely nutritional or in the turnover of cellular protein. As a result, the best characterised proteinases are the mammalian digestive enzymes such as trypsin, chymotrypsin and pepsin. However, the versatility of proteolytic enzymes extends beyond digestive functions to important regulatory roles in many physiological process (Neurath, 1989). Proteolysis is limited by the accessibility of susceptible peptide bonds and this limited proteolysis can result in important post-translational modifications of proteins which can alter their biological functions. Proteolytic processing can occur co-translationally, post-translationally or both and will involve, depending on the system, different proteases. The best studied co-translational processing is the release of signal peptides by signal peptidase during the transfer of proteins across membranes (Wickner and Lodish, 1985). Post-translational processing accompanies the cleavage of superchains into their constituent functional proteins (e.g. protein hormones and growth factors), macromolecular assembly (e.g. collagen fibril formation, biosynthesis of picorna viruses or bacteriophage) and zymogen activations, including consecutive activations in blood coagulation and complement systems (James and Bradshaw, 1984; Neurath, 1986).

Proteases may also play important roles in the pathogenesis of microorganisms such as penetrating the host, in countering host defence mechanisms, in enzyme, toxin and elicitor processing and in nutrition during infection (North, 1982). Particularly well studied have been the proteases of human pathogens, such as *Candida albicans* in view of the proteinaceous intercellular matrix that they encounter (Monod *et al.*, 1994).

Limited proteolysis due to differences in susceptibility and accessibility of peptide bonds combined with the substrate specificities of proteolytic enzymes has made proteases a major tool in the study of proteins, in both sequence analysis and in the identification and isolation of functional domains of proteins of known structure (Walsh, 1986). Proteases have also been extensively studied because of their commercial importance in for example, baking, brewing, cheese manufacturing, meat tenderising and in washing detergents (Priest, 1984).

Extensive study of proteolytic enzymes has led to their classification into four mechanistic classes based on a comparison of active sites, mechanism of action and three dimensional structure (Neurath, 1989). These four classes are serine, cysteine, aspartic and metallo proteases and within these, six families of proteases have been identified to date (Table 4.1.).

Family	Representative protease (s)	Characteristic active site residues
Serine protease I	Chymotrypsin Trypsin Elastase Pancreatic kallikrein	Asp ¹⁰² , Ser ¹⁹⁵ , His ⁵⁷
Serine protease II	Subtilisin	Asp ³² , Ser ²²¹ , His ⁶⁴
Cysteine proteases	Papain Actinidin Rat liver cathepsins B and H	Cys ²⁵ , His ¹⁵⁹ , Asp ¹⁵⁸
Aspartic proteases	Penicillopepsin <i>Rhizopus chinesis</i> and <i>Endothia parasitica</i> , acid proteases Rennin	Asp ³³ , Asp ²¹³
Metallo-proteases I	Bovine carboxypeptidase A	Zn, Glu ²⁷⁰ , Try ²⁴⁸
Metallo-protease II	Thermolysin	Zn, Glu ¹⁴³ , His ²³¹

Table 4.1. Families of proteolytic enzymes. Reproduced from Neurath (1989).

The table only includes enzymes of known amino acid sequence and three dimensional structure, except that for rat liver cathepsins B and H. The number of residues (column 3) corresponds to the amino acid sequence of the enzymes listed in bold in column 2.

Each protease family has a characteristic set of functional amino acid residues which are arranged in a particular configuration to form the active site. Serine protease family I contains the mammalian serine proteases chymotrypsin, trypsin and elastase while family II contains the bacterial serine proteases such as subtilisin. While the two families share a common active site geometry and enzymatic mechanism they differ in amino acid sequence and three-dimensional structure. A similar situation exists with the metallo-proteases between the mammalian pancreatic carboxypeptidase and the bacterial thermolysin. Not all proteases fit into the above classification; examples include the collagenases, the aminopeptidases and the signal peptidases (Neurath, 1989).

The cleavage of peptide bonds by proteases is brought about by nucleophilic attack on the carbon-oxygen bond (either directly or mediated by a water molecule) assisted by the donation of a proton to the peptide nitrogen. In each protease class different amino acids fulfil the functions of nucleophiles and others act as proton donors (Neurath, 1984).

Serine proteases are the best characterised and most physiologically versatile protease family. Their active sites comprise the catalytic triad of Asp, His and Ser and catalysis proceeds *via* a tetrahedral transition state intermediate during both the acylation and deacylation steps of catalysis (Kraut, 1977). An ester bond is formed between the oxygen of serine and the acyl portion of the substrate, with release of the 'amino' portion of the substrate as the first product. Also important are two backbone -NH-groups that are available for hydrogen bonding to the developing oxygen anion from the carbonyl group of the peptide bond undergoing attack. Differences in substrate specificity can be related to amino acid substitutions in the primary substrate binding site and to minor differences in the secondary binding sites.

The cysteine proteases are characterised by cysteine as the major catalytic amino acid residue. The attacking nucleophile is the sulphur atom of a cysteine side chain. Catalysis proceeds *via* a thiol ester intermediate and is facilitated by the side chains of adjacent histidine and aspartic acid.

The catalytic site of the aspartic proteases contains two aspartic acids which are in close geometric proximity to each other (Neurath, 1989). In contrast to the serine and cysteine proteases, hydrolysis of the peptide bond is not *via* nucleophilic attack by a functional group of the enzyme. Instead these enzymes rely upon general acid/general base catalysis of the attack of a water molecule. The aspartic and metallo-proteases differ from the cysteine and serine proteases in that they do not form covalent intermediates. The metallo-protease active site usually contains zinc, with its three ligands of two glutamic acids and one histidine. Another glutamic acid side chain acts as the nucleophile, directly or with the participation of a water molecule.

One way of determining the mechanistic class of an uncharacterised protease is with protease inhibitors (Dunn, 1989b). These can be divided into two general classes (i) active site-specific, low molecular weight inhibitors that irreversibly modify an amino acid residue of the active site, and (ii) naturally occurring protein protease inhibitors, many of which behave as pseudosubstrates. The first group contain the serine protease inhibitors diisopropyl phosphofluoridate (Dip-F) and phenylmethanesulphonyl fluoride (PMSF) which react with the active site serine. Aspartic proteases are inactivated by diaxoacetyl compounds as well as by the acetylated pentapeptide pepstatin which acts as a pseudosubstrate. Metalloproteases are inhibited by metal ion chelators such as EDTA and 1,10-phenanthroline. However care must be taken with these as proteases from other classes may be activated or stabilised by metal ions. Cysteine proteases are inhibited by the peptide diazomethanes, the natural proteinaceous inhibitors known as the cystatins and the peptide epoxides such as E64 which is thought to alkylate the active site cysteine. The less specific sulphydryl alkylating agent, iodoacetic acid also inhibits cysteine proteases.

Natural proteinaceous protease inhibitors have been isolated from animal, plant and bacterial organisms where they play important roles in protease regulation and in defence. The presence of elevated levels in plants following wounding by insects and infection by plant pathogens suggests that these serve to inhibit their digestive proteases (Ryan, 1990). Protease production has been detected in many plant pathogenic fungi and bacteria, e.g. *Uromyces viciae-fabae* (Raucher *et al.*, 1995), *Colletotrichum lindemuthianum* (Ries and Albersheim, 1973), *Ustilago maydis* (Hellmich and Schauz, 1988), *Botrytis cinerea* (Movahedi and Heale, 1990b) and *Xanthomonas alfalfae* (Reddy *et al.*, 1971). Their absolute requirement for pathogenicity has not been determined in many cases although protease deficient mutants of *Xanthomonas campestris* and *Pyrenopeziza brassicae* are reduced in virulence (Dow *et al.*, 1990; Ball *et al.*, 1991). In contrast, protease mutants of *Cladosporium cucumerinum* and *Cochliobolus carbonum* were unaltered in pathogenicity (Robertson, 1984; Murphy and Walton, 1996). However, in both cases there was residual protease activity.

Protein plays an important role in plant cell wall structure, interacting with cell wall carbohydrates and providing cell wall stability. In some plant species protein can account for up to 15% of the cell wall where it serves both structural and enzymic functions (Showalter, 1993). There are five major classes of cell wall proteins some of which, such as the extensins, a group of hydroxyproline-rich glycoproteins, are produced at elevated levels during pathogenesis (Mazau, 1986; Showalter, 1993). If the proteases produced by invading fungi can degrade these proteins they may play an important role in penetrating the plant cell wall barrier. Proteases may also serve other important roles during infection such as countering host defences, for example degradation of the antifungal enzymes chitinases and glucanases (Mauch *et al.*, 1988). Alternatively they may be involved in processing toxins (Howard and Buckley, 1985), elicitors (van den Ackerveken *et al.*, 1993), and enzyme zymogens (Rypniewski *et al.*, 1994). They may be toxic *per se* by their action on host cell walls (Movahedi and Heale, 1990a) or on the host plasma membrane (Tseng and Mount, 1974). Proteases

may also serve a nutritional role, e.g. in the interaction between the endophyte *Acremonium typhinum* and its host grass (Lindstrom and Belanger, 1994).

Recently, a protease inhibitor has been identified in the incompatible interaction between *S.nodorum* and barley (Stevens *et al.*, 1996). Protease production by *S.nodorum* has been observed on wheat seeds (Sieber, 1989) and on skimmed milk agar plates (Lehtinen, 1993). Protease production by *S.nodorum in vitro* on wheat cell walls has also been detected here (Chapter 3) and elevated levels of protease activity were detected in infected plant tissue. The aim of this chapter is to characterise this protease activity, to determine whether the activity in infected tissue is produced by *S.nodorum* and to try to understand the possible role for this protease activity in pathogenesis.

4.1 RESULTS

4.1.1 Regulation of protease activity *in vitro*

Protease activity was detected in the culture filtrate of *S.nodorum* grown on wheat cell walls by assay with the general protease substrate azocasein at pH5 and pH8 (Chapter 3). The aim of this aspect of the work was to investigate the role of carbon and nitrogen in the regulation of this protease. Following starvation, established biomasses were transferred into a range of culture conditions and samples taken at 12, 24 and 48h were assayed against azocasein at pH 5 (Fig.4.1) and pH 8 (Fig.4.2). As observed previously (Chapter 3), protease activity, as assayed at pH5, was approximately half that detected at pH8. In both cases protease production was greatest in cultures grown on wheat cell walls, in the absence of an additional nitrogen source. Protease production assayed at pH8 in these cultures continued to increase until 48h, in contrast to that assayed at pH5 which appeared to peak at 24h. This suggested that at least two different protease activities may be present in the culture filtrate, rather than the pH5 activity merely representing a lower activity of a protease with a more alkaline pH optimum.

Both activities were reduced by ca. 50% in the presence of nitrate. Activity at pH8 was reduced at all three time points while that at pH5 was only reduced at 24h, which resulted in the loss of the peak in protease activity, apparently caused by nitrogen starvation. The addition of glucose as well as nitrate to cell wall cultures further reduced protease production but did not completely repress it, as reflected by either pH 5 or 8 activity. Growth of *S.nodorum* on 1% glucose in basal salts containing nitrate resulted in almost complete elimination of both protease activities. Protease production therefore appeared to be partially repressed by nitrogen and this repression was increased in the presence of glucose. Activity was either derepressed or induced in the presence of wheat cell walls.

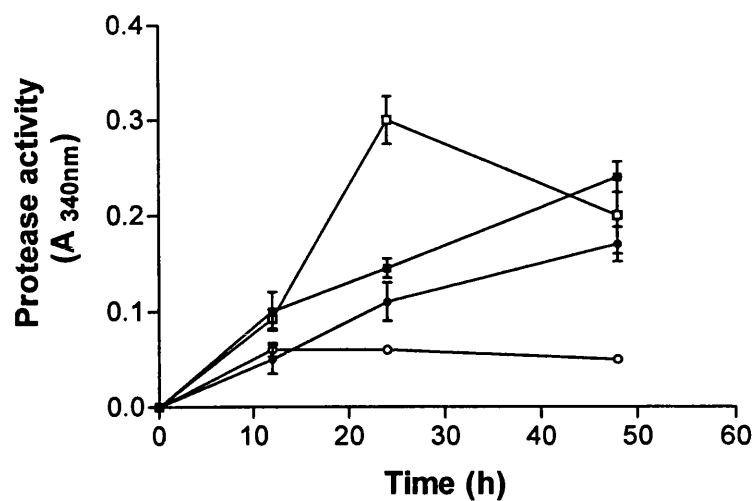


Fig.4.1 Regulation of protease activity *in vitro* as detected by assay against azocasein at pH5. CW+NO₃ (■), CW-N (□), CW+Glucose+N (●), Glucose+N (○).

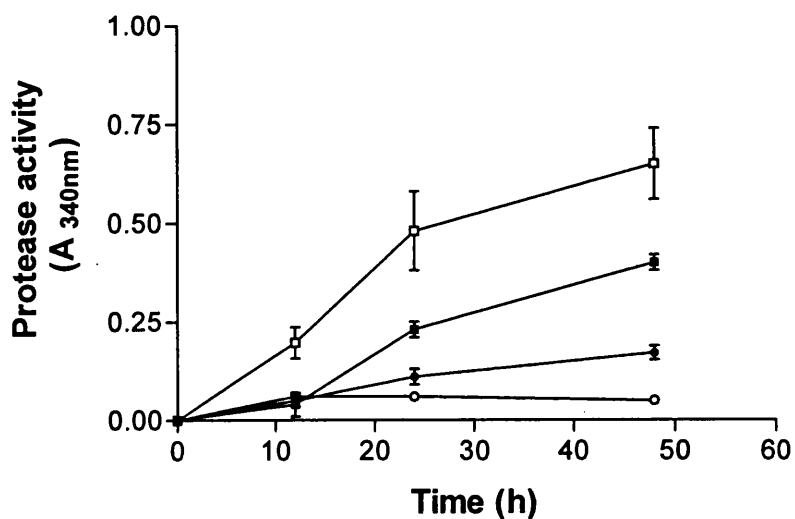


Fig.4.2 Regulation of protease activity *in vitro* as detected by assay against azocasein at pH8. CW+NO₃ (■), CW-N (□), CW+Glucose+N (●), Glucose+N (○).

4.1.2 Use of inhibitors to classify protease activity

The data on regulation indicated that the protease activity detected in fluids from cell wall grown cultures may be composed of at least two proteolytic enzymes with differing pH optima. Four main classes of proteases are known to exist: serine, cysteine, metallo and aspartic and these can be distinguished by the use of standard inhibitors specific for each class. There are a number of inhibitors specific for each class but those used in this study were: pepstatin (aspartic), iodoacetic acid (cysteine), PMSF (serine) and EDTA (metallo-proteases and metal ion activated proteases). Inhibitors were prepared in dH₂O except pepstatin and PMSF which were prepared as 1mM and 200mM stocks respectively in DMSO. 24h wheat cell wall (-N) culture filtrate was mixed with each of the four inhibitors for 30min before assay with azocasein at pH5 and pH8 (Fig.4.3). None of the inhibitors had any detectable effect on the pH5 activity and pepstatin and iodoacetic acid had no effect on the pH8 activity. However, ca. 50% reduction in activity at pH8 occurred with PMSF and a 10% reduction with EDTA. These data indicated that the pH8 activity was a serine protease, possibly more trypsin-like than subtilisin-like as indicated by incomplete inhibition with PMSF. Only weak inhibition of activity by EDTA indicated that there were no metallo-proteases present, but alternatively the serine protease activity may have a metal ion requirement. The absence of effect from any of the inhibitors used on pH5 activity was unexpected and hence does not place this activity in any protease class. The lack of influence of PMSF on pH5 activity again confirmed that this was not merely a reduced level of the pH8 activity.

4.1.3 Detection of an aspartic protease

The inhibitor studies detailed above indicated the presence of a serine protease but of no proteases from other classes. However, an activity detectable at pH5, apparently distinct from that at pH8 suggested that at least one other class may be present. At this time information was received from the PhD CASE sponsors,

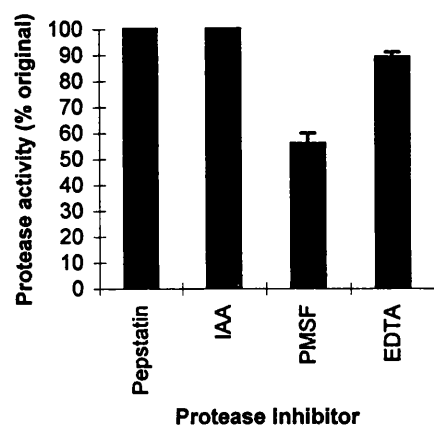


Fig.4.3. Effect of protease inhibitors on crude culture filtrate. Assayed against azocasein at pH8. No effect on activity assayed at pH5 was observed. Error bars represent the standard deviation about the mean of three replicates.

AgrEvo UK Ltd, regarding an aspartic protease gene isolated from *S.nodorum* by degenerate PCR (details withheld here). The failure to detect any aspartic protease activity during the inhibitor studies above suggested that the azocasein assay may not be suitable for detection of the aspartic protease, either because it is unable to act on this substrate, or because aspartic proteases generally have acidic pH optima and the assay pH of 5 may have been too high. Azocasein is not soluble at lower pH so it was not possible to test this theory. It also raised the possibility that this assay was not sensitive enough to detect the effect of pepstatin on the aspartic protease activity or that the pepstatin had been ineffective at inhibiting this activity. The latter was unlikely given that pepstatin is one of the most specific and effective protease inhibitors (Dunn, 1989b). An alternative assay was employed, based on that of MacDonald and Odds (1980). Each assay contained 600µl of 1% BSA in 50mM sodium citrate, pH3.2, and 200µl of culture filtrate. After 2h at 37°C 400µl of 10% (w/v) trichloroacetic acid was added to precipitate undigested protein and the tubes stored on ice for 10min. Following centrifugation at 11,000 xg for 10min the absorbance of the supernatant was measured at 280nm and corrected for background against a time-zero control in which trichloroacetic acid was added prior to the enzyme. Assay of 24h cell wall culture filtrate (-N) detected aspartic protease activity by this assay ($A_{280} 0.214 \pm 0.01$). To confirm that this was an aspartic protease, 1µM pepstatin (final concentration) was added and was found to inhibit completely this activity. This demonstrated that pepstatin was effective at inhibiting this protease suggesting that the azocasein assay at pH5 had failed to detect this activity.

Regulation of this aspartic protease activity was then investigated using the MacDonald and Odds (1980) assay, as before by transfer of starved, established biomasses into a range of culture conditions with samples taken at 24 and 48h (Fig.4.4). These cultures included BSA, gelatin and skimmed milk powder to test the induction of this protease by these different proteins. These were chosen in view of the apparent inability of the aspartic protease to degrade azocasein and the gelatin in a zymogram used below (section 4.1.4.2). Aspartic protease activity

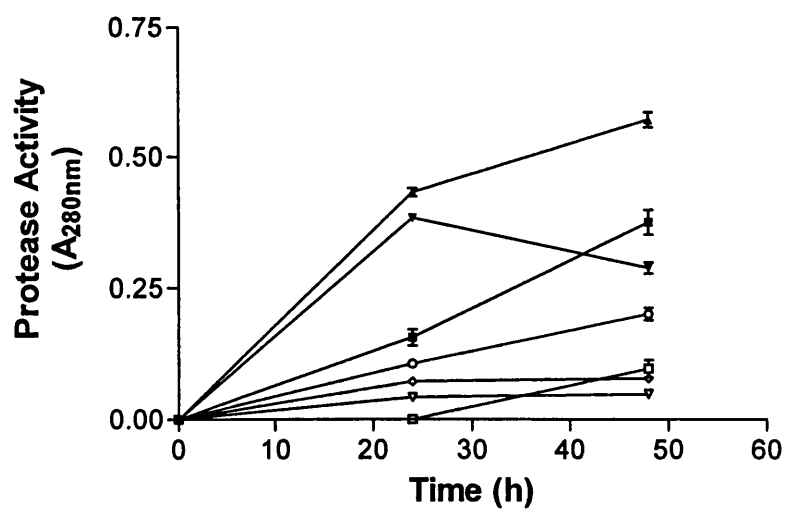


Fig.4.4. Regulation of aspartic protease activity in liquid cultures of *S.nodorum*. Established biomasses of *S.nodorum* were starved for 12h before transfer into a range of culture conditions. — \blacktriangledown Skimmed milk -N — \blacksquare CW + NO₃

— \blacktriangle CW + NH₄ — \blacktriangleright CW -N — \square CW + G -N — \circ Collagen -N
— \diamond BSA -N

No activity was detected in cultures grown on (Gelatin -N), (-C-N), (CW+G+NO₃), (+NH₄-G), (+NO₃-G), (G-N), (G+NO₃), (G+NH₄).

was absent in derepressed cultures containing no carbon or nitrogen source, and in the following cultures: (+NH₄-Glucose), (+NO₃-Glucose), (Glucose-N), (Glucose+NO₃), (Gelatin-N), (CW+Glucose+NO₃ or NH₄) which were also tested. It appears therefore that the aspartic protease is induced only in the presence of a protein source, and not by derepression of either carbon or nitrogen. Wheat cell wall cultures in the absence of an additional nitrogen source showed a peak in activity at 24h. This peak was not observed in cultures containing nitrate and activity was reduced by ca. 50% at this time. Production was not completely repressed by nitrate and by 48h, cultures with and without nitrate had very similar activity. Glucose was found to be more repressive than nitrate suggesting this enzyme is more strictly regulated by carbon. No activity was observed in cell wall cultures with additional glucose at 24h, but by 48h low levels could be detected. In cultures where nitrate was also present, production was repressed over the whole 48h. This contrasts with the regulation of the azocasein pH5 activity, which again indicates that this probably represents a different activity. Effective repression of the aspartic protease was only achieved therefore in the presence of both nitrate and glucose. In contrast to nitrate, the presence of ammonium apparently enhanced aspartic protease production. Ammonium was included because this is generally more repressive to fungal proteases than nitrate (Marzluf, 1993). Instead, ammonium appeared to increase aspartic protease production compared to cultures without additional nitrogen.

Induction of the protease was found to require relief from either carbon or nitrate repression and the presence of a protein source. Maximum induction of the aspartic protease was observed on wheat cell walls. At 24h collagen had only induced production to a third of that detected on wheat cell walls but by 48h the levels were similar. In contrast, BSA and skimmed milk powder only induced very low levels of aspartic protease production, approximately 25% of that observed on wheat cell walls. Gelatin failed to induce any aspartic protease production.

4.1.4 Isoelectric focusing determination of protease isoforms

The work detailed above had determined that at least two classes of protease activity were present in wheat cell wall grown cultures. To determine whether this protease activity comprised one or more different isoforms, proteases in cell wall grown culture filtrates were separated on the basis of isoelectric point (pI) by two different methods of isoelectric focusing; Rotofor™ preparative isoelectric focusing and gel isoelectric focusing.

4.1.4.1 Rotofor™ Preparative Isoelectric focusing

Dialysed 48h culture filtrate of *S.nodorum* grown on wheat cell walls in the absence of additional nitrogen source was separated by Rotofor isoelectric focusing using 1% ampholytes (BioRad) in the pH range 3-10. Once the voltage had stabilised, after approximately 4h, fractions were harvested and the pH, protein content and protease activity of each fraction determined (Fig.4.5). Protease activity was assayed with azocasein at pH 5 and 8 and with the BSA aspartic protease assay at pH 3.2. No protease activity was detected by assay with azocasein at pH5. A single peak at pH 9.2 was detected by assay with azocasein at pH8, suggesting that a single isoform of this extracellular activity exists in cultures. Two major peaks of aspartic protease activity were detected by assay at pH 2.6 and 4.5. The exact pI of these proteases could not be determined by Rotofor separation as this is purely a preparative step and pIs quoted are the pH of the fraction containing the activity. It does however give an indication of pI.

It was concluded from this work that a single serine protease with a high pI and two aspartic proteases with acidic pIs are present in wheat cell wall grown culture filtrates. This work clearly demonstrated the inability of the azocasein assay to detect the aspartic protease.

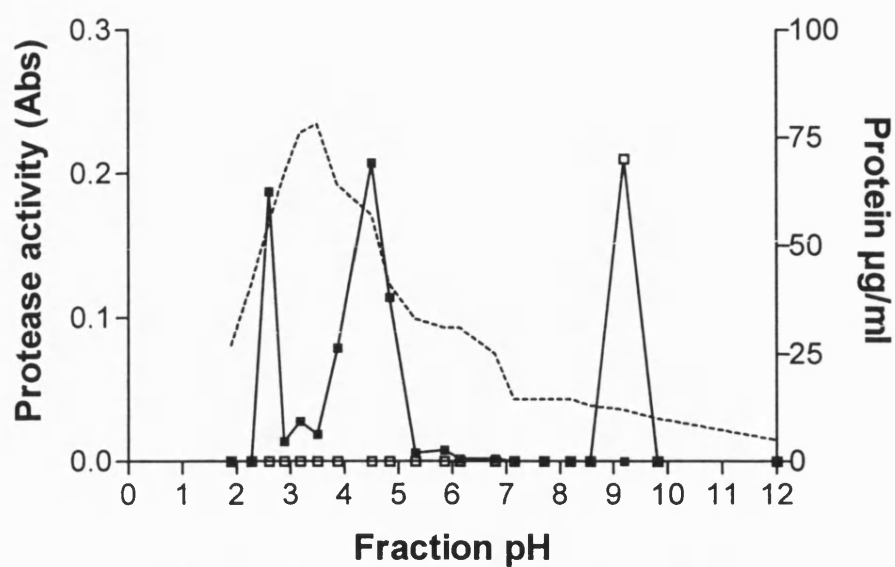


Fig.4.5. Rotofor separation of 48h cell wall culture filtrate. Fractions assayed against azocasein, pH8 (□) (A_{304nm}) and 1% BSA at pH3.2 (■) (A_{280nm}). Protein (-----). No activity was detected with azocasein at pH5.

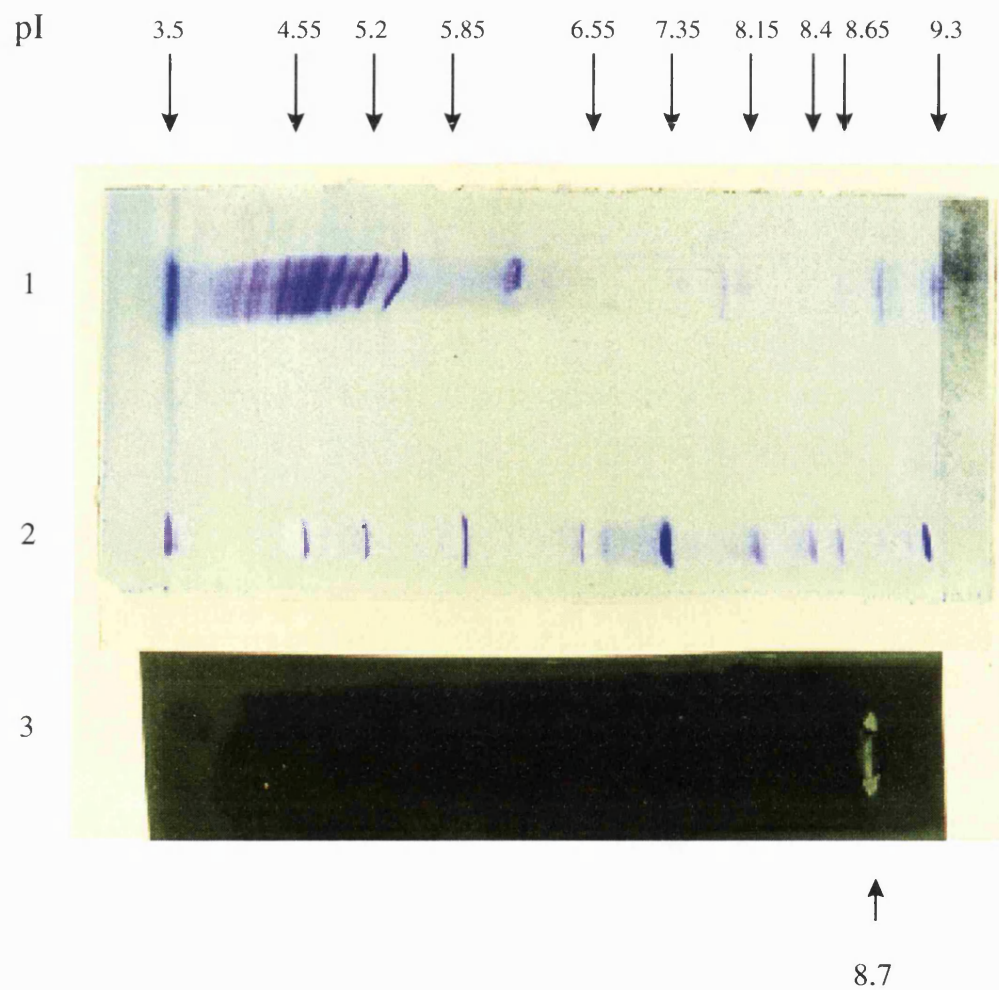


Fig.4.6. Isoelectric focusing gel of 48h cell wall grown culture filtrates of *S.nodorum*. Lane 1 culture filtrate, lane 2 pI marker, lane 3 X-ray film overlay showing a clearing zone corresponding to proteolytic degradation of the gelatin coating.

4.1.4.2 Gel isoelectric focusing

A more accurate determination of pI can be obtained by separation of culture filtrate proteins using gel isoelectric focusing followed by comparison to a series of known protein standards. This was performed using gels in the pH range 3.5-9.5, and following separation, protease activity was detected by overlaying the gel with X-ray film, previously soaked in water for 10min. Despite different incubation times of 15min to 24h only a single isoform of protease activity with a pI of 8.7 was detected by this method (Fig.4.6), similar to that detected on the Rotofor by azocasein at pH8. No acidic isoforms were detected, suggesting that the aspartic protease is unable to degrade gelatin; this was supported by the failure of gelatin as a sole source of carbon to induce this enzyme (Section 4.1.3).

Hence, a specific pI was only determined for the alkaline, serine protease. Both the Rotofor and the gel isoelectric focusing clearly demonstrate the limitation of using only one substrate for the detection of different proteases.

4.1.5 Partial purification and characterisation of an alkaline protease

The alkaline serine protease appeared from the isoelectric focusing results to be the major protease activity produced on wheat cell walls. Given that AgrEvo UK Ltd were working on the aspartic protease gene cloning, work here focused instead on the alkaline serine protease, especially as this appeared to exist as single isoform. The fact that this protease had an alkaline pI facilitated purification by cation exchange chromatography. Initially, a partially purified preparation was achieved using basic chromatography equipment. Later, a pure preparation of the protease was generated using higher resolution columns (Section 4.1.6).

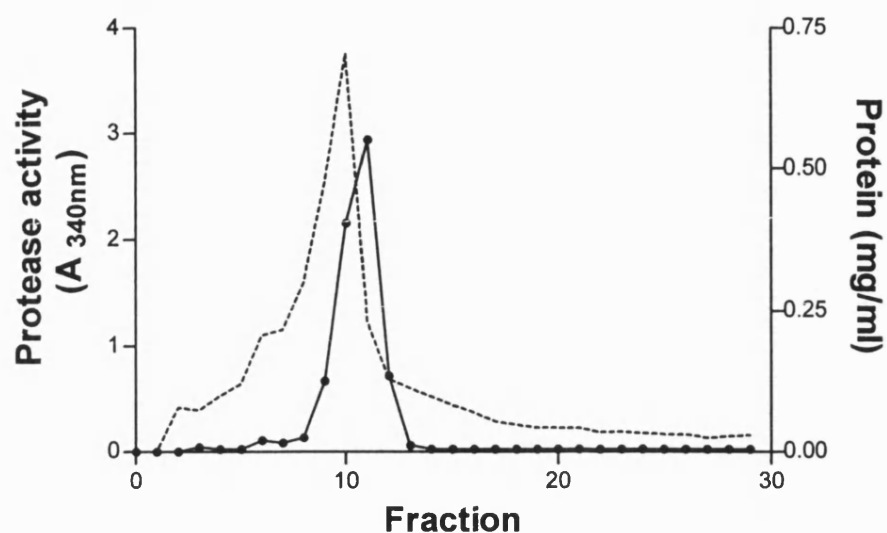


Fig.4.7. Partial purification of an alkaline protease from *S.nodorum*. 48h concentrated cell wall grown culture filtrate was loaded onto an S-column in MES at pH 6.0. Proteins were eluted over a 0-250mM NaCl gradient in the same buffer. Protease activity was detected using azocasein at pH8.0.

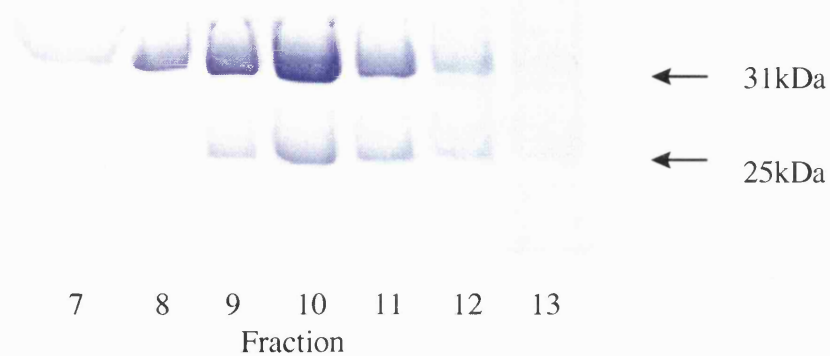


Fig.4.8. SDS-PAGE of S-column fractions containing protease activity as assayed by azocasein at pH8. Crude 48h *S.nodorum* cell wall grown culture filtrate was loaded onto a 10ml S-sepharose column in 25mM MES pH6.0 and eluted over a 0-250mM NaCl gradient (Fig.4.7).

A 10 fold concentrated sample of 48h cell wall culture filtrate was prepared by precipitating protein to 80% saturation with ammonium sulphate. A partial purification of the alkaline protease activity was achieved by fast-flow cation exchange chromatography of this extract. A single peak of protease activity (vs. azocasein) was eluted from the S-Sepharose column with a 0-250mM NaCl gradient (Fig.4.7). No activity was detected in the flow through. The activity did not however, correspond to the major protein peak and SDS-PAGE of the active fractions revealed two major proteins of ca. 25 and 31kDa (Fig.4.8). Further inhibitor studies on this partially purified protease revealed that this was a trypsin-like protease. The activity was not inhibited by pepstatin or iodoacetic acid as was observed in the crude extract (Section 4.1.2). A 15% inhibition of activity was achieved by EDTA, suggesting that the protease has some metal ion requirement, as found previously. Also repeatable was the partial inhibition (35%) by PMSF, indicating that this may be a trypsin-like protease. Trypsin-like proteases are known to be only weakly inhibited by PMSF compared to subtilisin-like proteases (Gebhard *et al.*, 1986; Powers and Harper, 1986). To determine if this was the case, the trypsin inhibitors aprotinin, leupeptin, soybean trypsin inhibitor and turkey egg white trypsin inhibitor were also included and all of these resulted in 70-80% inhibition of activity, confirming that this was indeed a trypsin-like protease (for full inhibitor characteristics on the pure protease see section 4.1.12). Trypsin-like proteases are active against Arg/Lys residues in the p1 position (Kraut, 1977) and a number of nitroanilide substrates exist for detection of trypsin-like proteases. The partially purified extract was assayed with the trypsin substrate Ben-Phe-Val-Arg-NA and found to be active. Knowing that this protease acted on a more facile, specific substrate, complete purification to homogeneity of the trypsin-like protease was followed using this substrate instead of azocasein at pH8. This assay also enabled quantification of protease activity.

4.1.6 Purification of a trypsin-like protease (SNP1)

The partial purification attempts had revealed that the alkaline protease activity was a trypsin-like protease that could be eluted from a fast-flow cation exchange column over a 0-250mM NaCl gradient. This work was repeated using a similar column, only now on an FPLC system (Pharmacia) with a gradient of 0-175mM NaCl in 25mM MES pH6.0. Trypsin-like protease activity was detected in a single peak, fraction 8 (Fig.4.9), by assay with the Ben-Phe-Val-Arg-NA substrate. This fraction was then buffer exchanged into 50mM HEPES-MES-Na Ac at pH 4.5 before further cation exchange separation on a BIOCAD Sprint system, using a higher resolution column. The protease was eluted with a 0-200mM NaCl gradient in the same buffer and activity was found in fractions 3-7 (Fig.4.10). Protease activity appeared as a single peak during both steps suggesting it was a single enzyme. SDS-PAGE revealed that peak fractions from the second cation exchange chromatography step contained a single major band of 25 kDa. Purification was monitored by SDS-PAGE (Fig.4.11). The protein content and the activities of the protease at the various stages of the purification procedure are given in Table 4.2 which shows a 417 fold purification was achieved.

Purification step	Protein mg/ml	Volume ml	Total Protein (mg)	Activity/ μ l (nkats)	Total activity (μ kats)	Specific activity/ mg (μ kats)	Purification (n-fold)
Culture filtrate	0.3	100	30	3	300	10	
Ammonium Sulphate	2.5	10	25	30	300	12	1.2
FPLC	0.4	2.5	1	46.7	116.7	117	11.7
Biocad	0.004	1.5	6 μ g	16.67	25	4167	417

Table 4.2. Purification summary for the isolation of an alkaline protease from the culture filtrate of *S.nodorum*

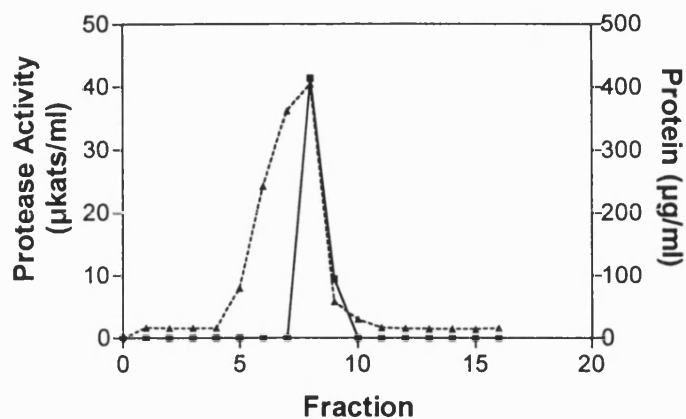


Fig.4.9. Elution profile of adsorbed proteins from *S.nodorum* culture filtrate on S-Sepharose chromatography in 25mM MES pH6.0; eluted with a linear salt gradient of 0-175mM NaCl. Protein (---), protease activity, $\mu\text{kats/ml}$ (■)

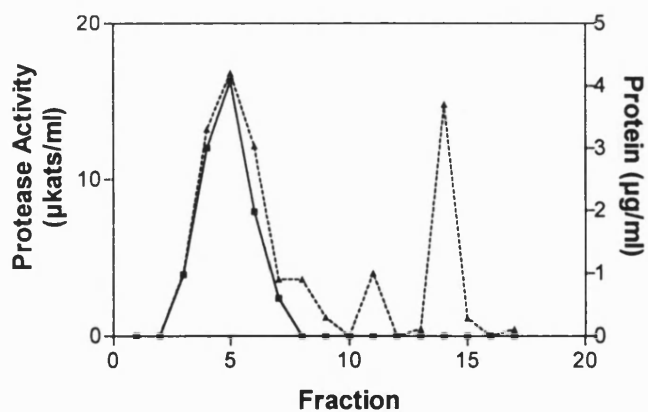


Fig.4.10. Elution profile of adsorbed proteins showing protease activity from S-Sepharose on FPLC (Fig.4.9) on an HS20 cation exchange column in 50mM HEPES-MES-NaAc pH4.5; eluted with a linear salt gradient of 0-200mM NaCl. Protein $\mu\text{g/ml}$ (----), protease activity, $\mu\text{kats ml}^{-1}$ (■)

4.1.7 N-terminal sequence

Peak fractions from the cation-exchange BioCad step were run on SDS-PAGE, blotted onto PVDF membrane and stained as described in materials and methods, and by Packman (1993). N-terminal sequencing of the 25kDa band confirmed that it was a trypsin-like protease. Sequence alignment with three other fungal trypsins is shown in Fig 4.12. The protease showed 94.1% identity with a trypsin-like protease from *Fusarium oxysporum* (Rypniewski *et al.*, 1993) and 84% identity with the ALP1 trypsin-like protease from *C.carbonum* (Murphy and Walton, 1996).

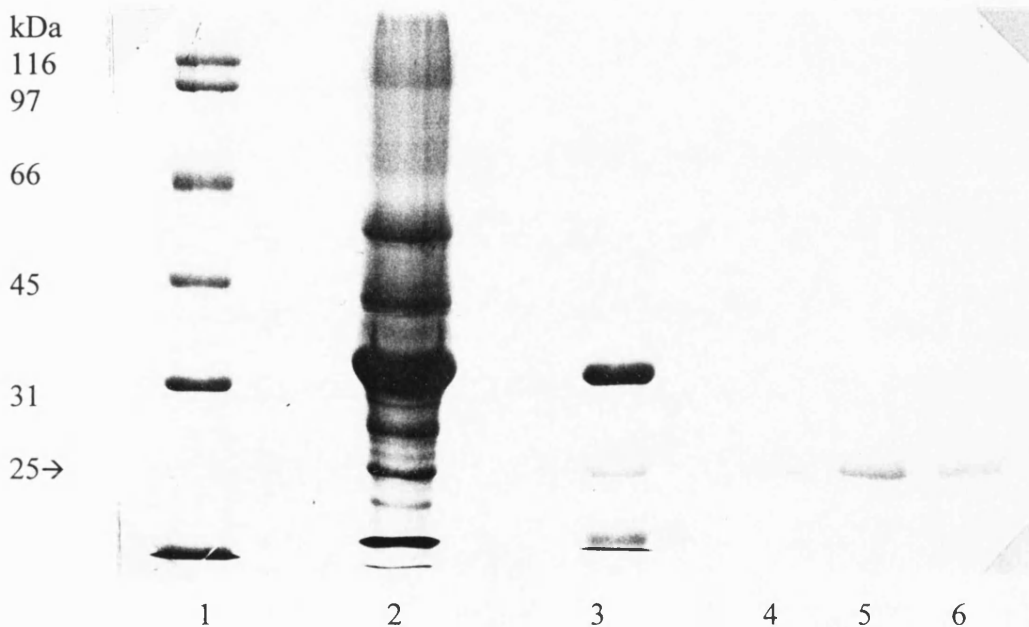


Fig.4.11. SDS-PAGE (12%) showing the purification of the trypsin-like protease (SNP1) secreted by *S.nodorum*. Lane 1, broad range molecular weight standards kDa (BioRad); lane 2, culture filtrate 48h after transfer onto wheat cell walls; lane 3, fraction 8 from FPLC cation exchange chromatography showing peak protease activity; lanes 4,5,6, 25kDa protein in fractions 4,5 and 6 from BioCad HS20 cation exchange chromatography showing peak protease activity.

<i>S.nodorum</i>	I V G G T T A S A G D F P F I V S I Q
<i>F.oxysporum</i>	I V G G T S A S A G D F P F I V S I S
<i>C.carbonum</i>	I V G G T T A A A G E Y P F I V S I Q
<i>V.dahliae</i>	K V G G V A A S A G D F P F K V S L Q

Fig. 4.12. Comparison of fungal trypsin N-terminal amino acid sequences from *S.nodorum*, *Fusarium oxysporum* (Rypniewski *et al.*, 1993), *Cochliobolus carbonum* ALP1 (Murphy and Walton, 1996) and *V.dahliae* VDP30 (Dobinson *et al.*, 1997).

4.1.8. Isolation and Characterisation of *SNP1*

The partial purification of the alkaline protease, (section 4.1.5), followed by inhibitor studies determined that this was a trypsin-like protease. Before pure protein and N-terminal sequence data were available two simultaneous approaches to cloning the gene encoding *SNP1* were taken. These were heterologous screening of a lambda library and PCR using degenerate primers.

4.1.8.1. *Heterologous library screen*

A *S.nodorum*, isolate LAW, Gem 11 lambda genomic library was screened as described in materials and methods with the *try1* (trypsin-encoding) gene from the entomopathogenic fungus, *Metarhizium anisopliae* (Smithson *et al.*, 1995). First round screening of approximately 15,000 plaques identified 10 clones which hybridised to this *try1* probe at 50°C. Of these, six showed strong hybridisation signals on the second round screen. DNA was purified from these six clones, restricted with *EcoRI*, *SacI* and *SalI*, fragments separated by gel electrophoresis and the resultant Southern blot membrane probed with the *try1* gene (Fig.4.13). All clones possessed at least one fragment which hybridised to *try1*, of which two of the strongest were a single *SalI* fragment from clones 1 and 6. These two bands, approximately 1,200bp and 1,600bp respectively were subcloned into pBluescript® and sequenced completely in both directions. No significant homology to any known sequences could be found in either of these clones. Simultaneous generation of a PCR product encoding the *S.nodorum* purified trypsin (see section 4.1.8.2) enabled these six clones to be 're-screened' on a Southern blot at 60°C. No specific hybridisation to any of these clones was observed, indicating that none of these clones encoded the purified trypsin-like protease.

4.1.8.2 PCR amplification of a SNP1 fragment

Initially, PCR was performed using degenerate primers based on the second half of the conserved fungal trypsin N-terminus, AG(DE)(FY)PFIV and to the active site sequence CQGDSGGP (as described in chapter 2). This generated a single product of around 580bp in size, consistent with that predicted from other fungal trypsin sequences (Fig.4.14). This product was excised from a gel and the DNA eluted before direct sequencing using the degenerate primers (Fig.4.15).

Sequence analysis of this product clearly demonstrated that this encoded a trypsin-like protease. Following N-terminal sequencing of the SNP1 protein a degenerate primer based on the SNP1 N-terminal amino acid sequence, IVGGTTA was used with a specific reverse primer, Tryp2 designed to the known sequence of the PCR product to generate a single major product of 515bp (Fig.4.16). The degenerate primers had generated a product of 580bp and specific primers based internally to that product produced a smaller product of 450bp. An intermediate product of 515bp was generated with the N-terminal primer and the reverse specific primer, indicating that the PCR product generated previously did encode the SNP1 protein. Confirmation of this was achieved once this product was sequenced and was shown to encode the rest of the N-terminal sequence SAGDFPFIV not used in the primer (Fig.4.17).

The PCR product contained an open reading frame (ORF) interrupted by one putative intron of 56bp. Comparison of the ORF to the Swissprot databases showed it to have a high homology to trypsin-like proteases (for full analysis see section 4.1.8.3). High stringency Southern blot analysis of *S.nodorum* genomic DNA detected the presence of only a single band in each digest, suggesting that the PCR product was derived from a single copy gene (Fig. 4.18).

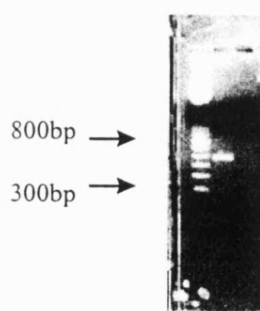


Fig.4.14. 580bp PCR product generated by degenerate PCR using primers to the second half of the conserved trypsin N-terminus and to the active site region on *S.nodorum* genomic DNA.

Tryp1 →
TTCTGCGGTGGTTCGCTGAAACGCCAACACCGTCCTGACCGCCGCTCACTGCGCTGTCGGACAGACT
 AAGACGCCACCAAGCGACGACTTGCGGTTGTGGCAGGACTGGCGGCGAGTGACGCGACAGCCTGTCTGA
 ← Out1
 F C G G S L L N A N T V L T A A H C A V G Q T
 GCTTCCAGCCTTCAGATCCGCGCTGGCTCTCTCgtaagacatgccatatttcaccatctcatccaactt
 CGAAGGTCGGAAGTCTAGGCGCGACCGAGAGAGcattctgtacggtataaagtggtagagtaggttgaa
 A S S L Q I R A G S L
 cccactaacaccccttcagAGCCGTACCTCCGGCGGAACCGTCGTCAAGGTGTCCTCCATCAAAGTCA
 gggtgattgtggggaaggtcTCGGCATGGAGGCCGCTTGGCAGCAGTTCCACAGGAGGTAGTTTCAGT
 S R T S G G T V V K V S S I K V N
 ACCCCTCGTACGCCGCCGGCACCTACAACAACGACGTTGCCATCATGAAGCTCGCCACCTCCATCCCCA
 TGGGGAGCATGCGGCGCCGCTGGATGTTGTTGCTGCAACGGTAGTACTTCGAGCGGTGGAGGTAGGGGT
 P S Y A A G T Y N N D V A I M K L A T S I P T
 CCAGCTCCACCATCAGCTACGCCAAGCTCGCCTCTTCTGGCTCCGACCCTGCCGCTGGCACCGGCCTCA
 GGTGAGGTGGTAGTCGATGCGGTTTCGAGCGGAGAACCGAGGCTGGGACGGCGACCGTGGCCGGAGT
 S S T I S Y A K L A S S G S D P A A G T G L T
 CCGTCGCCGGCTGGGGACCAACAGCTCCGGCGGCCAGTCCCTGCCCGTCAACCTCCTCAAGGTCGACG
 GGCAGCGCGGACCCCGTGGTGGTTCGAGGCCGCCGTCAGGGACGGGCAGTTGGAGGAGTTCCAGCTGC
 V A G W G T T S S G G Q S L P V N L L K V D V
 TGCCCGTCATCAGCCGCTCCAATTGCAACTCAA
 ACGGGCAGTAGTCGGCGAGGTGAACGTTGAGGTT
 ←Tryp2
 P V I S R S T C N S

Fig. 4.15. PCR product from genomic *S.nodorum* DNA and inferred amino acid sequence encoding a trypsin-like protease. PCR product was generated using degenerate primers to the second half of the conserved trypsin N-terminus and to the trypsin active site. The single intron is shown in lowercase and the sites of specific primers designed to this PCR sequence Tryp1 (forward), Tryp2 and OUT1 (reverse) are underscored. This PCR product was sequenced using the original degenerate primers; the sequence shown in bold corresponds to the 90bp overlap between the two sequences in which the double stranded sequence is confirmed. The remaining sequence has been sequenced in one direction only.

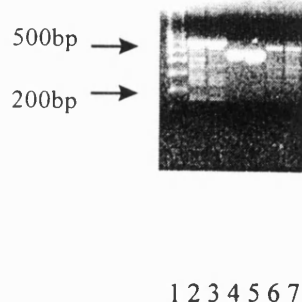


Fig. 4.16. PCR from *S.nodorum* genomic DNA used to confirm the previously isolated trypsin-like protease (Fig.4.15) encoded the purified trypsin-like protease SNP1. Lane 1, 100bp ladder; Lanes 2 and 3, degenerate primers based on trypsin conserved sequences (used previously to isolate a trypsin-like protease, Fig.4.14), 580bp; lanes 4 and 5, specific primers Tryp1 and Tryp2 internal to the trypsin-like protease (Fig.4.15), 450bp; lanes 6 and 7, degenerate primer based on SNP1 N-terminus sequence and Tryp2 specific primer based on the trypsin-like protease sequence (Fig.4.15), 515bp.

N-terminal

ATCGTTGGCGGAACCAACCGCCAGCGCTGGCGACTTCCCCTTCATCGTCTCCATCCAGCAGGGCGGAAGC
I* V G G T T A S A G D F P F I V S I Q Q G G S

CACTTCTGCGGTGGTTCGCTGCTGAACGCCAACACCGTCCTGACC
 H F C G G S L L N A N T V L T

Fig.4.17. N-terminal sequence of the PCR product (Fig.4.16) generated using a degenerate N-terminal primer based on SNP1 N-terminus and Tryp2, a specific primer to the trypsin-like protease generated by degenerate PCR (Fig.4.15). Sequencing was performed using the specific primer OUT1, based on the trypsin-like protease PCR product (Fig.4.15) to confirm that this encoded the purified protease SNP1. The isoleucine at the N-terminus of the mature protein is indicated * and the full N-terminus, identical to that obtained from the protein is in bold.

4.1.8.3 Isolation of full length SNP1 genomic sequence

A subsequent collaboration with Dr.Andy Bailey of IACR Long Ashton Research Station, Bristol has allowed the full length sequence of SNP1 to be obtained by re-screening of the lambda genomic library from the LAW isolate of *S.nodorum*, screened previously with the heterologous *try1* probe (Section 4.1.8.1). ³²P labelled PCR product (N-terminus to active site) encoding SNP1 was used to screen the lambda library. 15,000 plaques were screened and eight positives identified. On second round screening four positive plaques were identified and

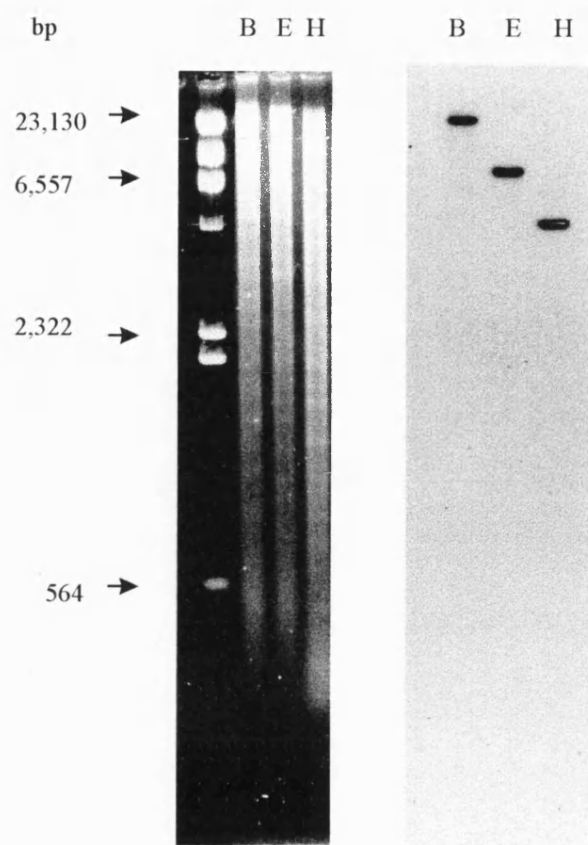


Fig. 4.18. Southern blot at high stringency demonstrating the 515bp PCR product hybridises to a single band. Isolated genomic DNA was cut with *Bam*HI, *Eco*RI and *Hind*III.

isolated DNA was digested with several different restriction enzymes and Southern blotted. Positive bands were cloned into pUC18 and sequencing identified that the whole gene was present on three of these clones. Several different *SalI* fragments were used as templates for sequencing with alternative templates used to confirm sequence across the *SalI* sites. The entire gene is present on a cloned 5.5-6kb *EcoRI* fragment. The full length sequence is shown in Fig.4.19.

SNP1 encodes a single ORF of 855bp containing a single putative intron of 56bp indicated by lower case letters. Intron location was inferred by disruption of consensus trypsin sequence and this is supported by the presence of a consensus 5' splice donor site GTANGA and a 3' splice acceptor site CAG characteristic of other fungal introns (Gurr *et al.*, 1987). Sequences typical of promoters of lower eukaryotes, TATAA and CAAC (Gurr *et al.*, 1987) were also detected. One TATAA at 911bp and two CAAC at 952 and 1012 bp upstream of the translation start site. No polyadenylation signal sequence ATAAA (Gurr *et al.*, 1987) could be identified downstream of the stop codon. Eight putative nitrogen responsive GATA sequence elements were identified upstream of *SNP1* three in the forward orientation (GATA) and five in the reverse orientation (TATC), overscored. Five putative carbon responsive sequence elements were also identified numbered c1-c5 and underscored, based on the consensus sequence SYGGRG. Finally two cAMP responsive elements were identified upstream of *SNP1* cre1 and cre2 which are underscored.

The DNA sequence obtained from the lambda library of isolate LAW was identical to the sequence of the PCR product obtained from isolate BS171 over the 520bp, including identical intron sequence. The N-terminal sequence was identical over the 16aa obtained from direct sequencing of the SNP1 protein. This confirms that the *SNP1* gene from isolate LAW encodes a protein identical over all regions sequenced to SNP1 purified from isolate BS171.

The translation start site of *SNP1* conforms to other consensus fungal translation start sequences in that there is an adenine at the -3 position (Gurr *et al.*, 1987). *SNP1* is predicted to encode a protein of 265 aa (29.4 kDa) which appears to be a preprotrypsin with a leader sequence of 36aa, subject to post-translational modification. A putative signal-peptide cleavage site was identified after Ala at aa 20, based on the (-3-1) rule according to Von Heijne (1986). This indicates a signal peptide of 20 aa which would leave a possible propeptide of 16aa between this signal peptide cleavage site and the consensus trypsin N-terminal sequence. Further proteolytic cleavage of this propeptide would result in a mature protein of 229aa (25.4 kDa) which is in agreement with the size of SNP1 estimated by SDS-PAGE. 80% of the amino acids in the signal peptide are hydrophobic suggesting that the protein is translocated to the endoplasmic reticulum. The sequence alignment of SNP1 with other trypsins from filamentous fungi indicates that there is significant identity with *F.oxysporum* (64%), *C.carbonum* (56%) and *M.anisopliae* (55%) (Fig.4.20). SNP1 contains the catalytic triad common to all serine proteases His-77, Asp-122 and Ser-218 (Neurath, 1984). The sequence surrounding the active site was also conserved as observed in all the other fungi except *M.anisopliae try1*.

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1  GTCGACGGGTGAATTTGCGGGACGGTGTTTCCGTCTCCTGCACGCCTGATCGCGCGATGC
61  GATGCAAAGTGGCACCTGCAAAGAAGCAAAGGACCGTCTGAGGAGGCATCCGGACAAC
121 CCGACCATCTCGTTCGAATGAAACGCCAGCACGTGATAGACCACAGGACTTGCCTCCTCC
181 CTATTCCTGCCTCATTCAGTAGCTGTCCAATGCGAGCGGCTGCACTTGAAGTGTCGAAG
      c1
241 CCAGCCCGCCTGTGGTGTGTTTGAAGATCCCGCCTCCACTGGGCATGATCGCCACCATGGC
      c2  c3
301 CCGGGGCTTGATTGACTACGCTACGCTGATGCCAAGCACCAATGTCTACGTGCAACTGC
      cre1
361 CTTTCTGGGCGCTGACCCGCTCGCTAAGTCGCATCACGTCAAGTCTCTACACCAACCAC
421 GCGCCGAGGCGTATCTTGTCTCCTGTTACTGCAATATAAGAAAAGCTTGGTATTCTCTTG
      c4
481 CCAAGAAGGCAGCGTGAAGATCATTGACCCACGTCCGATCGCCTACTCCGAGTATGGCA
      cre2
541 GGTCTGCCTGTTTCATGATTTGTACCACGGTTTGCTCATGGACGTCAACCATGTTATCGC
601 TCTTGATCGACAGTGTCTAGCATGATTATGCAGTCTACTATACGGCGTGGCCGTCTTCG
661 GCTGCTTTGCCCGCGAAACTTTGCAGATATGTCCGGGAATGGCCGCGCCTACATAGATCT
721 ACACAGGCCTTCTACACTGCACGGCGCCCGAAAGCAGATCTAACACGTCTGATTACACTC
781 GCGAGACCTCGCCACCAGGCCTCACAAGGCAGAAGACGCGGCGCGGAATTACTCGTGGC

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841 TGC GTGAAAGAGGTCG GCAACGAAACAGTTCTG CACTACGCTTAGCATAGCGCCGAAAAG
901 TCTCCTAATATCGGCCGTGGCATTAAAGGACTACACGGGAACTAGACGATCCACTTCGCCA
961 ACTTCCTCTTTGGCACTGTCACAGGCTACCCTAGAGCGCGCTTAAGCGACGGCAGATAAG
1021 TACCACTTCATCGGAGGCAAGGCACAACATGGACCTACATGTCCCCACTAAGCCAAAGAA
1081 GGTACTTGTAACTTTAGCAGCTCCAATCACTTCTCTGCCCAGACGTGATTAGACCCTATC
1141 TGGAAGGACCTGATCGTCCCCTGGCCTAATGACCTTCGAGCATCTACGACGACAGGACAG
1201 GAAGGTTGATGGACATCAGATGAAGCCATACACCATGTAGCACGCAACTGAAGTACAAAT
1261 ATCAGGGCCTCAACCCTCTCTTTGCAAACCAGTCCTTCACTACAACCTCTCGCTACAGACA
1321 CCTTCCAGCCAGCCAGCTTCAGACCTACCCTCAGCCTCATTCAAGATGCACTTCACAGAC
M H F T D 5
1381 GTCCTCATTTGCCCTCGCGGCTCCCGCTCTCATCAGCGCTGCGGCTATCCCCAGGACCCT
V L I A L A A P A L I S A A A I P Q D P 25
1441 GAGTTCCTGAGGGCGAGTTCCCGAGGACCCCATCGTTGGCGGAACCACCGCCAGCGCT
E F P E G E F P E D P I*V G G T T A S A 45
1501 GGGCACTTCCCCTTCATCGTCTCCATCCAGCAGGGCGGAAGCCACTTCTGCGGTGGTTCTG
G D F P F I V S I Q Q G G S H F C G G S 65
1561 CTGCTGAACGCCAACACCGTCTGACCGCCGCTCACTGCGCTGTGCGACAGACTGCTTCC
L L N A N T V L T A A H C A V G Q T A S 85
1621 AGCCTTCAGATCCGCGCTGGCTCTCTCgtaagacatgccatatttcaccatctcatcaa
S L Q I R A G S L 94
1681 cttcccaactaacaccccttccagAGCCGTACCTCCGGCGGAACCGTCGTCAAGGTGTCTCT
S R T S G G T V V K V S S 107
1741 CCATCAAAGTCAACCCCTCGTACGCCGCCGGCACCTACAACAACGACGTTGCCATCATGA
I K V N P S Y A A G T Y N N D V A I M K 127
1801 AGCTCGCCACCTCCATCCCCACCAGCTCCACCATCAGCTACGCCAAGCTCGCCTCTTCTG
L A T S I P T S S T I S Y A K L A S S G 147
1861 GCTCCGACCTGCGGCTGGCACCGGCCCTACCGTCGCGGCTGGGGCACCACCAGCTCCG
S D P A A G T G L T V A G W G T T S S G 167
1921 GCGGCCAGTCCCTGCCCCTCAACCTCCTCAAGGTCGACGTGCCCGTCATCAGCCGCTCCA
G Q S L P V N L L K V D V P V I S R S T 187
1981 CTTGCAACTCCAACCTACGGCAGCGGACGCATCACGACGGCCATGTTCTGCGCTGGCCTGT
C N S N Y G S G R I T T A M F C A G L S 207
2041 CCCAGGGTGAAAGGACTCGTGCCAGGGTGACTCTGGCGGCCCCATCATCAACACCTCTT
Q G G K D S C Q G D S G G P I I N T S S 227
2101 CGCGCGTGCAGGTTGGTGTGGTTTCTTGGGGTGAGGGTTGCGCGTCTGCGGGTGCGCCTG
R V Q V G V V S W G E G C A S A G A P G 247
2161 GTGTCTACTCCAACCTTGGGCCAGAGCAGCCTTGCGAGCTTTGTTGCTGCCAACTTGTA
V Y S N L G Q S S L A S F V A A N L 265
2221 CACACTTTATGAATGGATGAGTTATGGTTCTAAGTTGGAGAAGGAAGTGGTCTTGGTTGG
2281 TTGGAAGTGTATGTCTCAGCCACAGATTAATTCTTGGTAAATACTTTTTCAAATGCG

2341 TCATTGAATTGAAAATTGTTTTTTCTACACCTGAAAGCTGGGCTTTTTTTGCAAGTGAT
 2401 GCCTCTATGGTGATGATTGTTTGAATACTCTTGGAGCGGGCGCTGCAGGGGCAGTGGGAG
 2461 CGTCTCCAGACGTGGCTGAAGTCGGGGTTACGCAGTTCTTCTTCAGCGGCTTGAACACCC
 2521 TGTTTGACGGAAGTGGATCCTGCTTGGCGGCCGTTCCATCCCCTCGTCATCTTCGTTTG
 2581 GGGGATTGTGTGCACCGCCGCCCAATGAGGGCGTCTCTCACCTAGTCTCCATTTTGGA
 2641 TTCGGCTCAGATGCAGCTGGCTCATCTTCATCACTGATATTTGTCCGGTGGATCGCTCAG
 2701 ATACGAGTCCGAGTCCTCGAGGATCTCCACGGAAAGGCTGAGAGGTGATGCTGTTGCTGC
 2761 AGCTGCATCTCTTGCCGCATACGCAGCTGCCAACATTCTCTCGCCTTCTGCTGTGTCCTG
 2821 GAGGTCGTGAGATCGAGAGAGGAGATGTCTGACTCTGGAGGGTCATCCGTGTTGGTATT
 2881 CAATGTGCCCCGAGCAGCGAAGCTCTCAGCACCTAGATTGAAGTCGTTGCCAGGAGTTTG
 2941 CGATGCTGGGGCTAGAGCTGGGGTACTGTTTCATCTTGATTGCAGCGGCTTCAGGAGCGTC
 3001 GTTCATGTTGCTGTCTATGTTGGTGTGACTACGGGCTTGACTTGGGTGGTCGACTCAGG
 3061 AGGGGCGTCTTCGATACCCTCTATGTCAGATTGATCGGAAGGCTGTGCTAGAAGCACGGT
 3121 TTCAACCTCAGTAGGCAAAGAATCAGTGATGAACTCGGCAGTCCTGACAGGAAGTGCTAA
 3181 AGAAGGATCGATTGTTTCGTCAGCGACAGGTCCTAAGGCGGACCCGAAGACTTCGCGAT
 3241 CAAACCGTTGGTAGCAGGATGCTCTCGAGTAGGTACTTCAAGCTGGCTAGAAGGCTGTTC
 3301 CAGAATCTCAGCCTCCACTGCATCCGGCGAAGAAGTAGTGGTGTTTTTGGTAGTCTCATC
 3361 AGGAATTGATGAGGAAGATTTCGACAGATTTCGTTGAAAACAGGCCTTGGGCGGAATTCGCG
 3421 AGACGCCTTGACTAGGGCGTTGGTAGCAGCGCTTGCTCGCTCCAGACTCTTTGTTTCCT
 3481 TTGATCTTTGTGTTTACGTACATATCTTACTTTGTAACTCGCACAGCGGTAGTCTTGCT
 3541 CTTCTGCGCTGAAGAGTTTTGTGCCACTGGAGTGCGGTCGAC 3582

Fig.4.19. Genomic DNA and inferred amino acid sequence of the *Stagonospora nodorum* *SNP1* gene encoding a trypsin-like protease. The single intron is shown in lowercase, potential CREA binding sites (SYGGRG) are underlined and numbered c1-5, GATA sites for possible AREA binding are overscored and possible cyclic AMP response element binding sites (consensus TGACGTCA) are underlined and numbered cre1 and cre2. The isoleucine at the N-terminus of the mature protein is indicated * and the catalytic triad residues His (77), Asp (122) and Ser (218) are in bold.

S.nod	-----IVGGTTASAG	10
F.oxys	rvkfasvvalvaplaaaapqeipn-----IVGGTSASAG	34
C.carb	trfqsmittaalpavlslsaptqpwwddvpedsIVGGTTAAAG	40
M.anis	melksllavylplcaaplaaarpasnavf-IVGGSPAAAG	39
S.nod	DEPFIVSIQQGGSHEFCGGSLLNANTVLTAAHCAVGQTASS	50
F.oxys	DEPFIVSISRNGGPWCGGSLLNANTVLTAAHCVSGYAQSG	74
C.carb	EYPFIVSIQLGGRHNCGGTLINGNTVVTAAHCSVSSAIGG	80
M.anis	EEPFIVSTLLNGRHWCGGVLLNANTVLTAAHCVESTPAIS	79
S.nod	----LQIRAGSLSRTSGGTVVKVSSIKVNPSYAAGTYNND	86
F.oxys	----FQIRAGSLSRTSGGITSSLSSVRVHPSYSGN--NND	108
C.carb	sinnVAVRVGSLSANSGGQVIKVSIIIIHPSYQASTSNND	120
M.anis	-----QVRAGSLAHASGGVVANISITPHPKYEG--LGVD	112
S.nod	VAIMKLATSIPTSSTISYAKLASSGSDPAAGTGLTVAGWG	126
F.oxys	LAILKLSTSIPISSGNIGYARLAASGSDPVAGSSATVAGWG	148
C.carb	IAIWKLSSTVTAGGNIGFASLAASGSDPASGSTTSVAGWG	160
M.anis	MAILKLSTPIEANGTI--VRHIARGSDPVGGADATVAGWG	150
S.nod	TTSSGGQSLPVNLLKVDVPVVISRSTCNSNYGSGR----IT	162
F.oxys	ATSEGGSSTPVNLLKVTVPVIVSRATCRAQYGTSA----IT	184
C.carb	ATREGGGANN-ALLKVSVPVIVARSTCVSNYNAVGLT--VT	197
M.anis	DLEYAGQA-PEELOKVTVPVVDRAATCSAAYQAIPNMpnIT	189
S.nod	TAMECAGLSQGG-DSQGDGGPIINTSSRVQVGVVSWGEG	201
F.oxys	NQMECAGVSSGG-DSQGDGGPIDSS-NTLIGAVSWGNG	222
C.carb	TNMVCAGVTAGR-DSQGDGGPVDAN-KTLIGVVSWGTTG	235
M.anis	DAMECAGLKEGGQDACNGDGGPIDTETRVLIGVVSWGKYK	229
S.nod	CASAGAPGVYSNLGQSSLASFVAANL	229
F.oxys	CARPNYSGVYASVGA--LRSEIDTYA	248
C.carb	CARPNLPGVYSRVGT--LRSEIDQNA	261
M.anis	CAAPNAYGVYTRLGA--DIEFIKSHL	254

Fig.4.20. Multiple sequence alignment of fungal trypsin sequences aligned using Macaw 2.0.5. The aligned sequences are S.nod, SNP1 from *S.nodorum*; F.oxys, *Fusarium oxysporum* trypsin (Rypniewski *et al.*, 1993); C.carb, *Cochliobolus carbonum* trypsin ALP1 (Murphy and Walton, 1996) and M.anis, *Metarhizium anisopliae* try1 (Smithson *et al.*, 1995). Identical amino acids are highlighted.

4.1.9 Regulation of SNP1

Aspects of the regulation of SNP1 were determined by assay against the trypsin substrate, Ben-Phe-Val-Arg-NA under a range of culture conditions. This assumed that no other trypsin-like proteases were acting on this substrate, although none had been detected during purification stages.

Maximal activity was detected in cultures grown on wheat cell walls in the absence of any additional nitrogen source, with peak activity at 24h twice that of cultures containing nitrogen (Fig. 4.21). Collagen induced protease activity to ca. 50% of that on wheat cell walls at 24h but by 48h levels were similar. When nitrogen was added with wheat cell walls, as either NaNO_3 or NH_4Cl at 25mM, activity was reduced but not completely repressed; there was no apparent difference in the ability of nitrate or ammonium to repress. Protease production on wheat cell walls was repressed in the presence of glucose but by 48h activity was detected, presumably following glucose depletion. Repression by glucose was most effective in the presence of nitrogen; similarly, greater repression by nitrogen was observed in the presence of glucose, suggesting both are required to achieve complete repression. Protease production was not observed in cultures containing no protein source ie. (NH_4 -Glucose), (NO_3 -Glucose), (Glucose-N), (Glucose+ NO_3) or (-Glucose -N). Hence, production of SNP1 required the presence of wheat cell walls or collagen and the relief of either carbon or nitrogen repression, in particular carbon.

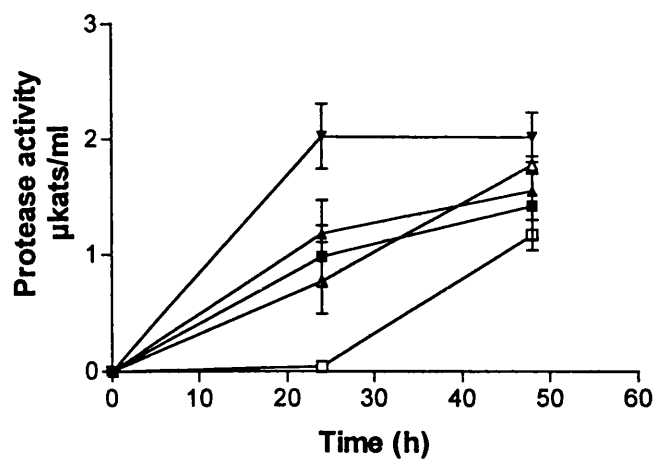


Fig.4.21. Effect of carbon and nitrogen on the regulation of SNP1. *S.nodorum* was grown for 48h in Czapek Dox-V8 complete medium, transferred into basal salts medium for a 12h starvation before transfer into basal salts medium minus nitrate and supplemented with a range of carbon and nitrogen sources. All carbon sources were at 1% and nitrogen is at 25mM. —■— CW + NO₃ —▲— CW + NH₄ —▼— CW -N —□— CW + G -N —△— Collagen -N
No activity was detected in cultures grown on (-G-N) or (CW+G+NO₃).

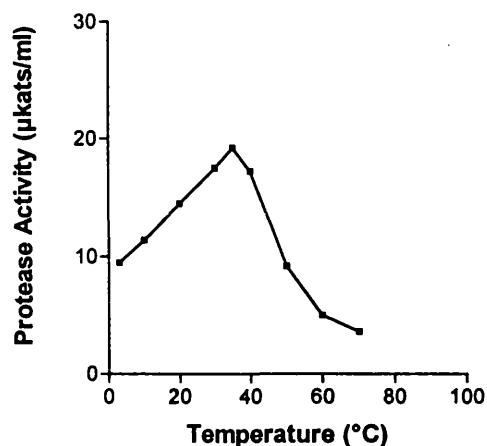


Fig.4.22. Temperature optimum for SNP1. Pure SNP1 was assayed over a range of temperatures with a 15min incubation time.

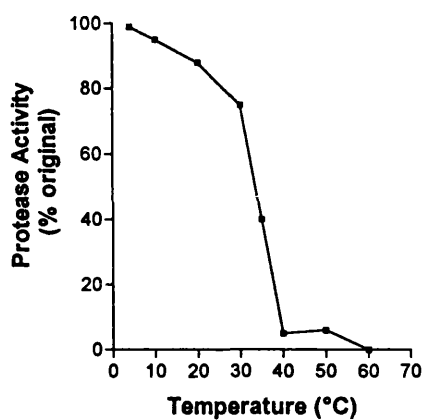


Fig.4.23. The effect of temperature on the stability of SNP1. Pure SNP1 was incubated at each temperature for 1h before assay.

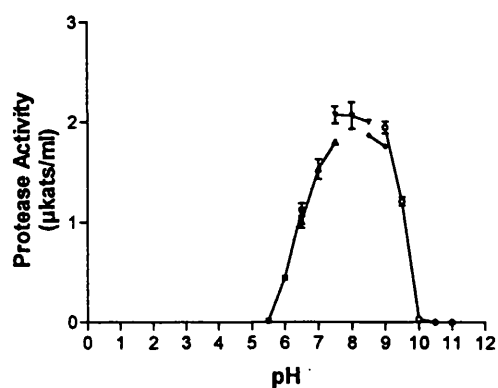


Fig.4.24. pH optimum for SNP1. Pure SNP1 activity was assayed over a range of pH values using five overlapping buffers to cover pH 5.5-11.
 —•— MES —•— Mops —•— Tris-HCl
 —•— Glycine —•— Caps.

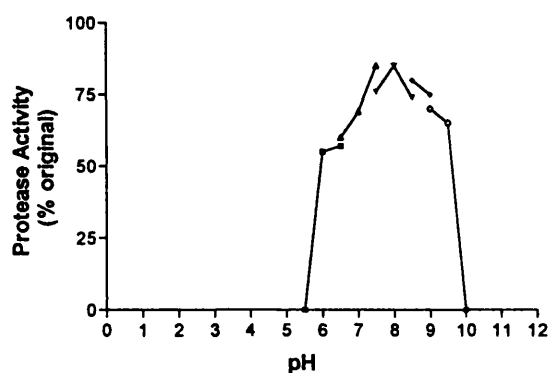


Fig.4.25. The effect of pH on the stability of SNP1. Pure SNP1 was incubated for 1h in the pH range 5.5-11 before assay. Buffers as for Fig.4.24.

4.1.10 Temperature and pH optima

SNP1 had a temperature optimum of 35 °C (Fig.4.22) but was most stable at 4°C after a 1h incubation period (Fig.4.23). Activity of pure SNP1 was assayed with Ben-Phe-Val-Arg-NA over a range of pH values using five overlapping buffers to cover pH 5.5 to 11. All at 50mM; MES pH 5.5-6.5; MOPS pH6.5-7.5; Tris-HCl pH7.5-8.5; Glycine-NaOH pH8.5-9.0; CAPS pH 9.5-11. It had a broad pH optimum over the range 7-9 but activity rapidly declined above and below this (Fig. 4.24). Consistent with this was maximum stability at pH8 (Fig. 4.25).

4.1.11 Metal ions

Pure SNP1 was extensively dialysed against several changes of 25mM MES pH6 and then incubated with a range of metal ions for 30min before assay. Metal ions were used in the concentration range 1.25 - 10mM. Enzyme activity was unaffected by MnCl₂, MgCl₂, NaCl, ZnCl₂ over the range 1.25mM-10mM or by PbAc and FeCl₃ over the range 1.25-5mM, but was inhibited by 85% and 60% respectively at 10mM. Protease activity was stimulated ca. 22% by calcium ions, optimally at 2.5mM. This calcium requirement is consistent with the enzyme being a trypsin-like protease.

4.1.12 Inhibition characteristics

Confirmation that SNP1 was a trypsin-like protease was also obtained with the use of standard inhibitors (Fig. 4.26). Pure SNP1 was mixed with inhibitor for 30min before assay against Ben-Phe-Val-Arg-NA; concentrations are final concentrations in reaction mixtures: Aprotinin (1μM), Leupeptin (1mM), PMSF (1mM), EDTA (10mM), Iodoacetate(100μM), Pepstatin (1μM), Soybean trypsin inhibitor (100μg/ml), Turkey egg white inhibitor (100μg/ml). All inhibitors were prepared in dH₂O except pepstatin and PMSF which were prepared as 1mM and 200mM stocks respectively in DMSO.

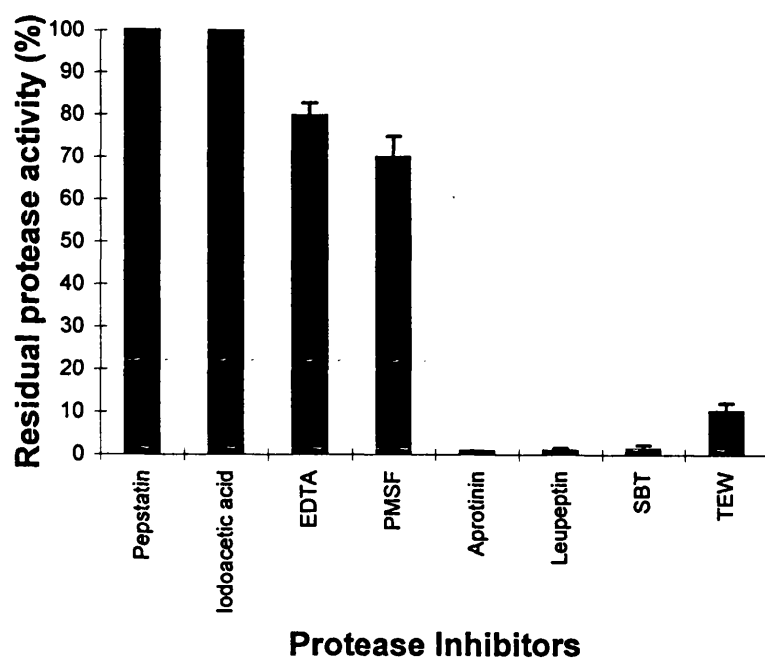


Fig. 4.26. Effects of protease inhibitors on pure SNP1 activity. Inhibitors were mixed with pure enzyme for 30min before assay.

SNP1 was strongly inhibited by aprotinin (99%) and leupeptin (99%) and weakly inhibited by PMSF (30%), characteristic of trypsin-like proteases (Gebhard *et al.*, 1986; Powers and Harper, 1986). Soybean trypsin inhibitor (SBT) and turkey egg white (TEW) trypsin inhibitor also showed activity against the protease with 98% and 90% inhibition respectively. EDTA also inhibited activity by 20%, consistent with the metal ion requirement of calcium; this also coincided with some loss of activity of protease after extensive dialysis. The cysteine and aspartic protease inhibitors iodoacetic acid and pepstatin were ineffective against SNP1.

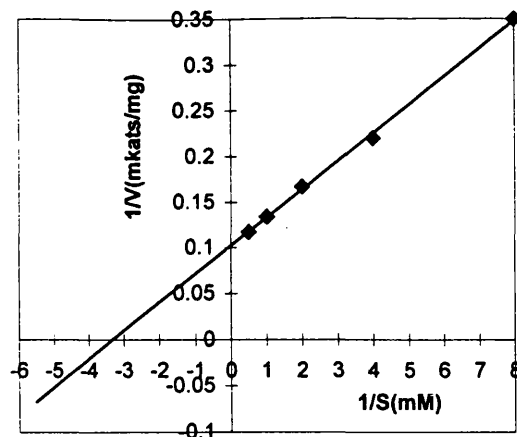
4.1.13 Substrate range and Kinetics

Pure SNP1 was assayed against a range of nitroanilide substrates to assess substrate specificity. It showed no activity against the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-NA or the subtilisin substrate CBZ-Gly-Gly-Leu-*p*-NA.

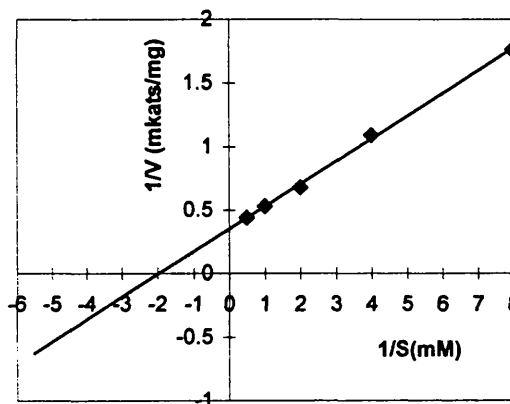
A kinetic analysis was performed with the pure protease against a range of trypsin nitroanilide substrates and the K_m and V_{max} calculated for each using the direct linear plot (Cornish-Bowden and Eisenthal, 1974). These were plotted by hand. For ease of presentation the Lineweaver-Birk plots are shown in Fig. 4.27, however differences in K_m and V_{max} are due to the difference in plot. The direct linear plot enables a more reliable calculation of these values. The substrate range used was 0.125-2mM with a fixed enzyme concentration of 0.02 μ g (Table 4.3)

Substrate	K_m (mM)	V_{max} (mkats/mg)
Ben-Phe-Val-Arg-NA	0.25 ± 0.017	7.3 ± 0.21
D-Val-Leu-Arg-NA	0.30 ± 0.023	7.5 ± 0.17
Ben-Pro-Phe-Arg-NA	0.63 ± 0.038	1.5 ± 0.11
D-Val-Phe-Lys-NA	0.39 ± 0.019	2.8 ± 0.03

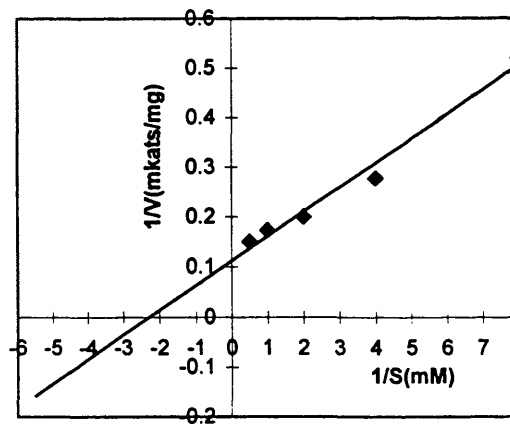
Table 4.3. Kinetic constants of substrates cleaved by SNP1.



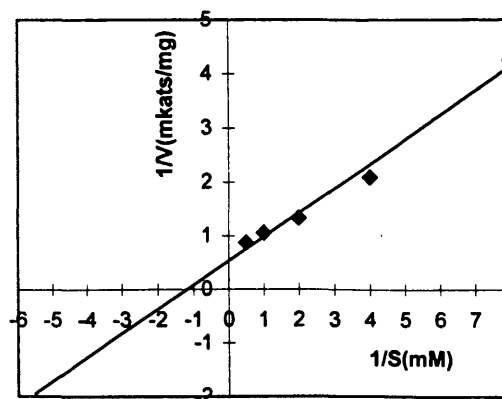
Ben-Phe-Val-Arg-NA
 $K_m = 0.285 \text{ mM}$
 $V_{max} = 10 \text{ mkats/mg}$



Ben-Val-Phe-Lys-NA
 $K_m = 0.5 \text{ mM}$
 $V_{max} = 3.3 \text{ mkats/mg}$



Ben-Val-Leu-Arg-NA
 $K_m = 0.4 \text{ mM}$
 $V_{max} = 9 \text{ mkats/mg}$



Ben-Pro-Phe-Arg-NA
 $K_m = 0.4 \text{ mM}$
 $V_{max} = 2 \text{ mkats/mg}$

Fig.4.27 Lineweaver-Burk plots for hydrolysis of a range of nitroanilide substrates by SNP1

SNP1 showed a preference for the substrates Ben-Phe-Val-Arg and D-Val-Leu-Arg. It was least active against Ben-Pro-Phe-Arg but higher activity was observed when the Arg was replaced by Lys against the substrate D-Val-Phe-Lys. The ability to cleave at the carboxyl side of both Arg and Lys is indicative of a trypsin-like protease.

4.1.14. Effect of SNP1 on wheat cell walls

SNP1 was found to be induced by collagen (section 4.1.9), and was capable of degrading azocoll (data not shown), a hydroxyproline rich glycoprotein. Hence it was possible that SNP1 may be able to degrade plant cell wall protein. The ability of pure SNP1 to degrade wheat cell wall proteins was investigated by detecting the release of hydroxyproline containing wall fragments following incubation of 5mg of wheat cell wall with SNP1 for 12h. 5mg of wheat cell wall was washed for 1h with agitation in 50mM Tris-HCl, pH8. Washed walls were then centrifuged at 11,000 xg for 15min and the buffer removed. This was replaced with 950µl of fresh buffer and 50µl of test (a range of SNP1 concentrations) or control sample prepared in the same buffer. Samples were incubated with agitation at 25°C for 12h before centrifugation at 11,000xg for 15min. Supernatants containing degradation products were removed and transferred to 11cm pyrex tubes with teflon lined lids. Proteins in the sample were then hydrolysed in 6M HCl (final concentration) for 20h at 110°C to release free hydroxyproline. Samples were then lyophilised before assay for hydroxyproline using the methods of Kivirikko and Liesmaa (1959). Solutions used for assay were: A=180µl bromine dissolved in 50ml of ice-cold 1.25 M NaOH; B=16% Na₂SO₃; C=5% *p*-dimethylaminobenzaldehyde in propan-1-ol; D=6 M HCl. Samples were resuspended in 300µl of dH₂O and cooled on ice. 300µl of ice-cold A was then added, shaken vigorously and incubated on ice for 10min. 15µl of B was then added followed by 300µl of C and finally 150µl of D, samples were shaken between each addition. Samples were then incubated at 95°C for 2.5min followed by 10min at 20°C. Absorbance was read at 560nm and compared to a standard curve of hydroxyproline over the range 0.1-2.0µg.

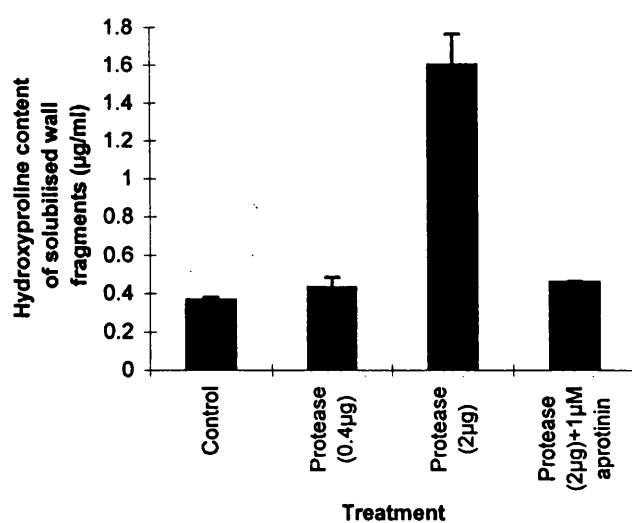


Fig. 4.28. Release of hydroxyproline containing wall fragments from wheat cell walls following incubation with pure SNP1 for 12h. Following incubation for 12h solubilised proteins in the supernatant were hydrolysed with 6M HCl at 110°C and free hydroxyproline released determined. Error bars represent the standard deviation about the mean of three replicates

Two different concentrations of pure SNP1 were used, the lowest of which, 0.4µg of protein, corresponding to 1.6µkats, had no significant effect on the wall compared to controls (Fig.4.28). The addition of a concentrated protease solution corresponding to 2µg of protein (8µkats activity) resulted in an approximately 3.5 fold increase in hydroxyproline containing proteins being released from the wall. This was equivalent to a total of 1.6µg of hydroxyproline, approximately 40% of that present in the wall (total hydroxyproline in the wheat cell wall was determined following hydrolysis of 5mg of cell wall in 6M HCl for 20h at 110°C and found to be 4µg). Addition of the trypsin inhibitor, aprotinin, reduced levels of hydroxyproline release to that of controls. This clearly demonstrates that pure SNP1 alone can degrade the wheat cell wall.

4.1.15 Detection of SNP1 *in planta*

The ability of SNP1 to degrade wheat cell walls suggested that this enzyme may play a role in wall degradation during infection of the host by *S.nodorum*. To investigate whether SNP1 could be detected *in planta* during infection, the characteristics of the trypsin-like protease activity detectable in infected and control plant material was investigated. Protease was extracted from infected and control plants at days 2, 7 and 14 after inoculation as described in materials and methods for extraction of enzymes. Forty g of each tissue was extracted and following protein precipitation by 80% ammonium sulphate, dialysed against 20mM MES pH6.0 and concentrated to 20ml in a solution of PEG 20,000 Mr.

Protease activity (vs. Ben-Phe-Val-Arg-NA) was detected in both control and infected plant material throughout the time course. Levels in extracts from infected plants were ca. twice that observed in controls (Table 4.4). Protease activity in the extracts from both control and infected plant tissue at day 7 was low compared to the other time points.

Time	Control A _{405nm}	Infected A _{405nm}
day 2	0.127/mg	0.340/mg
day 7	0.075/mg	0.182/mg
day 14	0.360/mg	0.675/mg

Table 4.4. Trypsin-like protease activity detected in control and infected plant material at 2,7 and 14 days after inoculation by assay for 15min with Ben-Phe-Val-Arg-NA.

The elution profile of the trypsin activity in both control and infected plant material from a cation exchange column was investigated by following the same purification method used in the initial purification attempt of SNP1 (Section 4.1.5). 10ml of each plant extract was applied to an S-sepharose fast flow cation exchange column (10ml) previously equilibrated in MES pH6.0. Proteins were eluted over a 0-250mM NaCl gradient and the protein content and trypsin-like protease activity in each fraction determined. Because of extremely low trypsin-like protease activity prolonged incubation with substrate was required. The elution profiles for each of the time points are shown in Fig.4.29 compared to the control elution profile for SNP1 from 48h cell wall grown culture filtrate. The presence in all six extracts of a peak in trypsin-like activity in approximately fractions 18-20 suggests that this is a plant trypsin-like protease which is increased in activity in infected plant material; typically this was more than twice that detected in control plant material. Extracts from infected plants possessed an additional peak of trypsin-like protease activity, not detected in control tissue. This peak, clearest in day 14 tissue, co-eluted with the *S.nodorum* SNP1 ca. fraction 11. A similar peak was observed at day 7, but at day 2 this peak was less clear; although it appeared to be present effective separation from the plant protease was not achieved. Overall, however it appeared that SNP1 was present in infected tissue at all three time points.

SDS-PAGE gels were run using fractions from control and infected plant material co-eluting with the fungal trypsin and examined for the presence of SNP1 (as evidenced from a 25kDa band). SDS-PAGE of day 14 and day 7

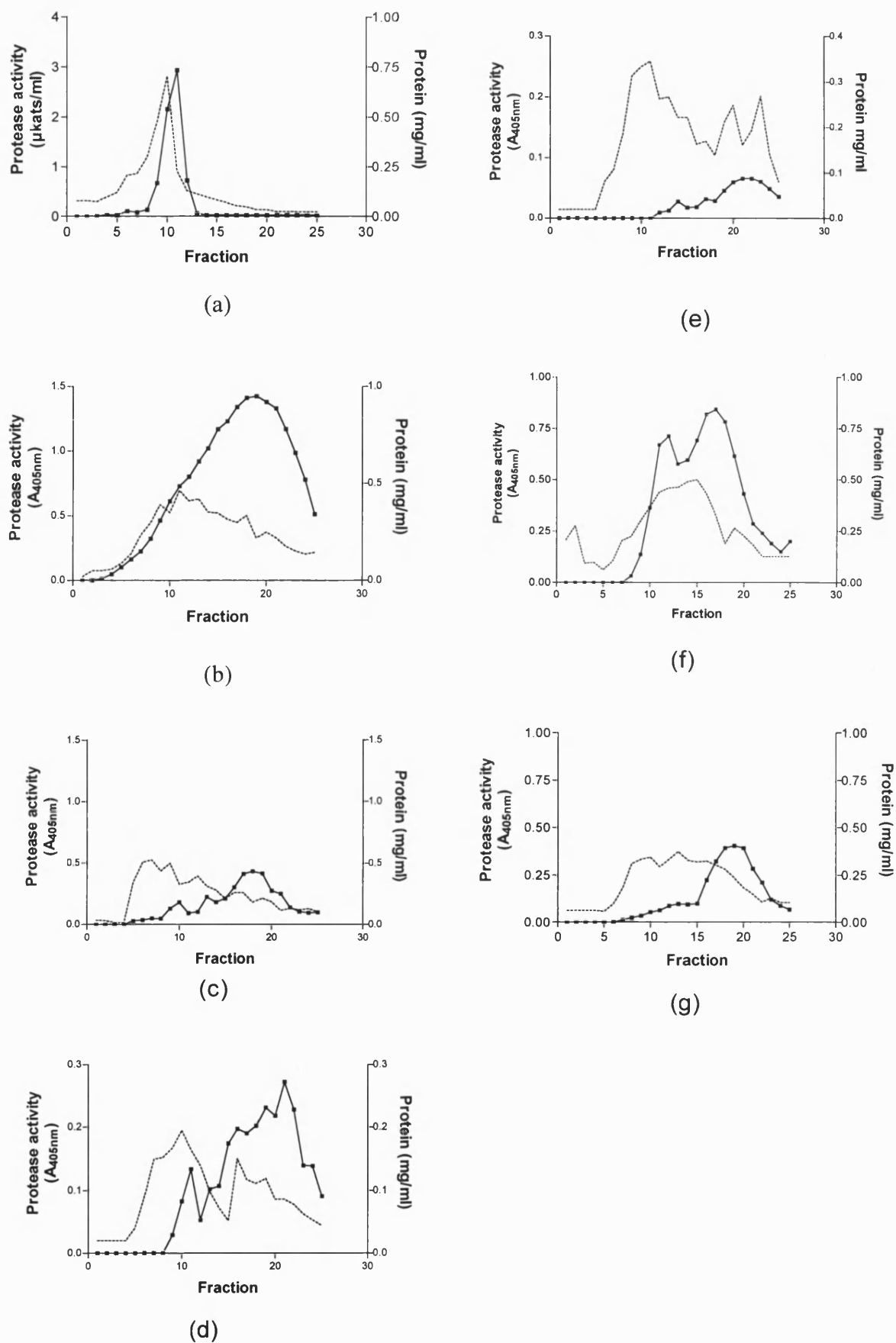


Fig.4.29 Elution profiles of infected and control plant protein extracts adsorbed onto an S-sepharose cation exchange column in 25mM MES pH6.0; eluted over a linear 0-200mM NaCl gradient. Protein mg/ml (----). Protease activity (-) is expressed as absorbance units following a 12h assay time. (a) Elution of *S.nodorum* culture filtrate. (b) Infected day 2; (c) control day 2; (d) infected day 7; (e) control day 7; (f) infected day 14; (g) control day 14.

extracts did not reveal a 25kDa band that could easily be distinguished from controls. In contrast, day 2 fractions did clearly show a band of 25kDa not present in control fractions (Fig.4.30), consistent with the presence of the fungal protease, SNP1. To further characterise the activity in these co-eluting fractions

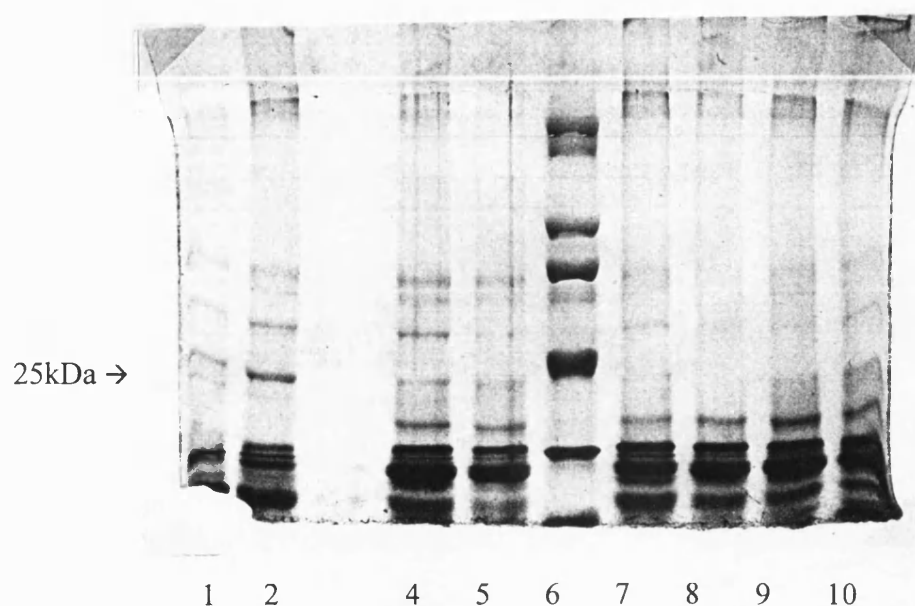


Fig.4.30. SDS-PAGE of S-column fractions from day 2 infected and control plant material co-eluting with SNP1. Lanes 1,2,4,5 fractions 9-12 infected day 2, lane 6 BioRad markers, lanes 7-10, fractions 9-12 control day2. Arrow indicates the presence of a 25kDa band in fractions 9 and 10 of infected tissue not present in controls.

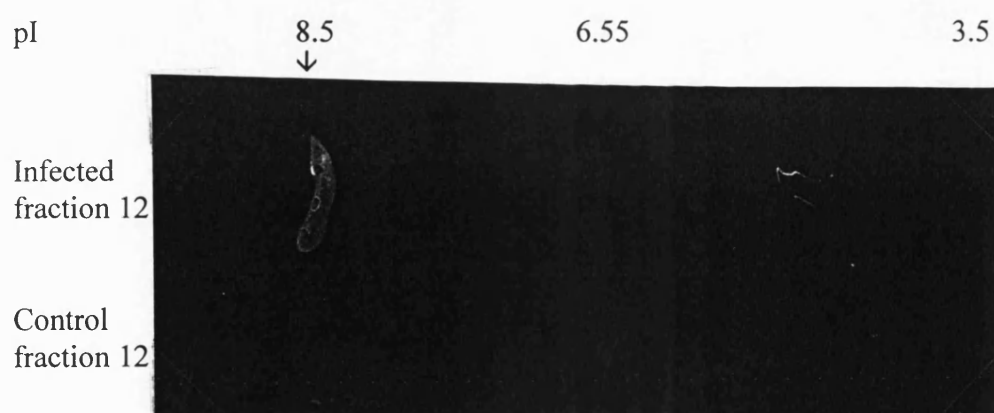


Fig.4.31. X-ray film overlay of infected and control S-column fractions from day 14 material. X-ray film was overlaid onto an IEF gel following isoelectric focusing of fraction 12 from both control and infected plant material. The blue clearing zone indicated by the arrow is a result of proteolytic degradation of the gelatin layer coating the X-ray film. The lighter zone around pI 6.55 is an artefact.

proteins were separated on an isoelectric focusing gel and subsequent gelatin overlay used to determine the pI of the protease activity present in these fractions. Because day 7 co-eluting fractions were low in protein and trypsin-like protease activity these were not used for IEF. Surprisingly, no activity was detected in day 2 fractions using the overlay method. Gelatin overlays were left in place for 24h in case any activity appeared but none was detected. However, following incubation with the gelatin overlay overnight, a clearing zone did appear in fraction 12 from day 14 infected tissue which was not present in the same fraction from control tissue (Fig.4.31). This protease activity had a pI of ca. 8.5, again consistent with the presence of SNP1 which has a pI of ca. 8.7.

Finally, activity in these co-eluting fractions was also completely inhibited by the trypsin inhibitors aprotinin and leupeptin, as seen previously for SNP1 (Section 4.1.12). Taken together this circumstantial evidence strongly implicated the presence of SNP1 during infection of wheat by *S.nodorum*.

4.1.16 Detection of SNP1 *in planta* by western blotting and both SNP1 and the aspartic protease in cirrus mucilage

To confirm the circumstantial evidence for the presence of SNP1 *in planta* during infection by *S.nodorum* Western blot analysis was performed using antibodies raised against pure SNP1. The ability of the antibody to detect SNP1 was confirmed by detection of 1µg of SNP1 by four different dilutions of the antibody (Fig.4.32). SNP1 was still just detectable at a 1/1000 dilution of the antibody indicating that the titre of the antibody was low. The specificity of the antibody towards crude culture filtrate is shown in Fig.4.33.

For detection of SNP1 in infected plant material a new, more rapid method of generating infected plant material was developed, as described in materials and methods. Detached leaves were inoculated by dipping into a spore solution of 1×10^5 spores/ml and material was collected at 24h, 2, 4 and 7 days after infection. No symptoms were observed at 24h; at 2days chlorotic spots appeared; by 4 days

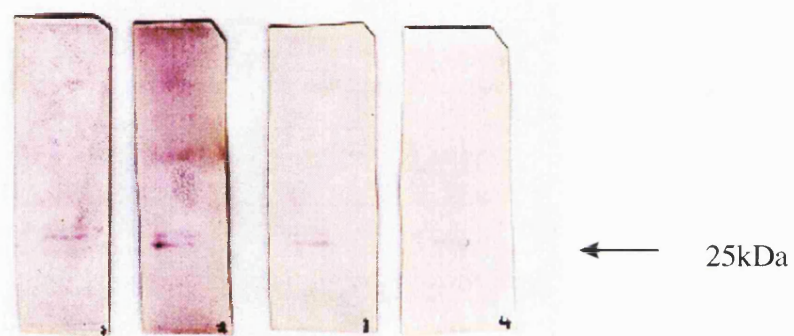


Fig.4.32. Detection of pure SNP1 with anti-SNP1 antibody. 1 μ g of SNP1 was electrophoresed using SDS-PAGE and blotted onto nitrocellulose. Blots were incubated for 1h with 1/10, 1/100, 1/500 and 1/1000 dilutions of the antibody and antibody binding detected with an alkaline phosphatase linked goat anti-rabbit antibody.

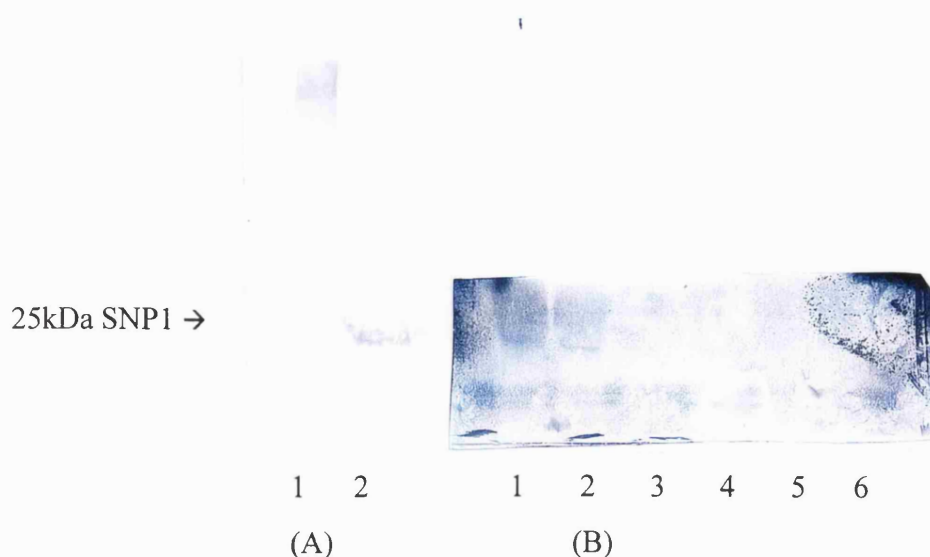


Fig.4.33. (A). Western blot of 100µg of cirrus mucilage (lane 1) and 100µg of 48h cell wall-grown *S.nodorum* culture filtrate (lane 2) probed with the anti-SNP1 antibody. Blots were incubated overnight in a 1/10 dilution of the antibody before detection with an alkaline phosphatase linked goat anti-rabbit antibody. (B). Western blot of control and *S.nodorum* infected plant material probed with the anti-SNP1 antibody as for (A). Lane 1, infected day 7; lane 2, infected day 4; lane 3, infected day 2; lane 4, infected 24h; lane 5, control day 7; lane 6, control day 2.

necrotic spreading lesions were present and by day 7 the leaves were moribund. Control plant material was harvested at day 2 and day 7. One mg of protein extract from infected and control plants was blotted onto nitro-cellulose following SDS-PAGE electrophoresis along-side 100µg of 48h 1% wheat cell wall culture filtrate as a control and 100µg of cirrus mucilage. Western blots were then probed overnight with a 1/10 dilution of the SNP1 antibody (Fig.4.33). No cross-reactivity with other bands in the crude culture filtrate was evident. The top half of the western blot from plant material was probed separately from the lower half because the high antibody concentration reacted non-specifically with abundant high molecular weight proteins, effectively reducing the available antibody. A 25kDa band, corresponding to the position of SNP1 was present at all time points in infected tissue but not in controls. The 25kDa band was present

24h after inoculation but was only at low levels at day 2. More SNP1 could be detected at days 4 and 7 as infection spread through the leaf. Detection of SNP1 in *in planta* extracts was only possible with a 1/10 dilution of the antibody, incubation in the antibody solution overnight and by loading a high concentration of plant protein extract (1mg) onto the starting gel.

The antibody also detected a faint 25kDa band, that looked slightly degraded, in the cirrus mucilage, collected from spores produced *in vitro*. Protease activity in cirrus mucilage had previously been detected by assay with azocasein (Chapter 3). Confirmation was made of a trypsin-like protease activity (vs. Ben-Phe-Val-Arg-NA), presumably SNP1. At the same time, aspartic protease activity was also detected (Table.4.5)

Enzyme	Activity detected in cirrus mucilage (per mg protein)
SNP1	575 nkats
Aspartic protease	0.24 (A 280nm)

Table 4.5. Detection of trypsin-like protease activity (vs.Ben-Phe-Val-Arg-NA) and aspartic protease (vs. BSA at pH 3.2) in cirrus mucilage.

4.1.17. Effect of inhibition of protease activity on disease

The detection of SNP1 in infected plant tissue and in cirrus mucilage, combined with its known ability to degrade the plant cell wall, suggested that this protease may play an important role during infection of the plant. Protease inhibitors and the SNP1 polyclonal antibody were used to investigate the effect on disease of inhibiting SNP1 and the aspartic protease.

4.1.17.1 Effect of protease inhibitors on infection

The trypsin inhibitors aprotinin and leupeptin were used to study the effect of inhibition of SNP1 during infection, and pepstatin was used to inhibit the

aspartic protease. These inhibitors were chosen because they all resulted in 99% inhibition of the appropriate enzyme activity *in vitro*. Inhibitors were mixed with spores for 30min before application to the leaf surface and symptoms assessed at 4 and 7 days after inoculation (Fig.4.34). Data were statistically analysed using the Mann-Whitney non-parametric test (Table 4.6). The effect of aprotinin at 1 μ M on disease symptoms compared to controls can be seen in Fig. 4.35.

After four days all three inhibitors had reduced infection; aprotinin at 1 μ M caused complete inhibition of disease symptoms, aprotinin at 10 μ M reduced disease symptoms by up to 70% and aprotinin at 100 μ M by up to 50%. Leupeptin was less effective, reducing symptoms by up to 40 and 30% for 1mM and 10mM respectively. Neither inhibition by aprotinin at 100 μ M nor leupeptin at 10mM caused significant reduction compared to controls. Pepstatin also showed a 50% inhibition of disease symptoms at day 4. By day 7, none of the inhibitors had significantly reduced disease compared to controls except for leupeptin at 1mM which still inhibited symptom formation by ca.30%.

Treatment	P value vs control (Day 4)	P value vs control (Day 7)
Aprotinin 1 μ M	0.0079 *	0.548
Aprotinin 10 μ M	0.0317 *	0.222
Aprotinin 100 μ M	0.0952	0.841
Pepstatin 1mM	0.0317 *	0.3095
Leupeptin 1mM	0.0317 *	0.0317 *
Leupeptin 10mM	0.0952	0.6905

Table. 4.6. Mann-Whitney (non-parametric) analysis of the effect of inhibitors on disease symptoms at days 4 and 7 after inoculation. Significance ($P < 0.05$ is denoted by *).

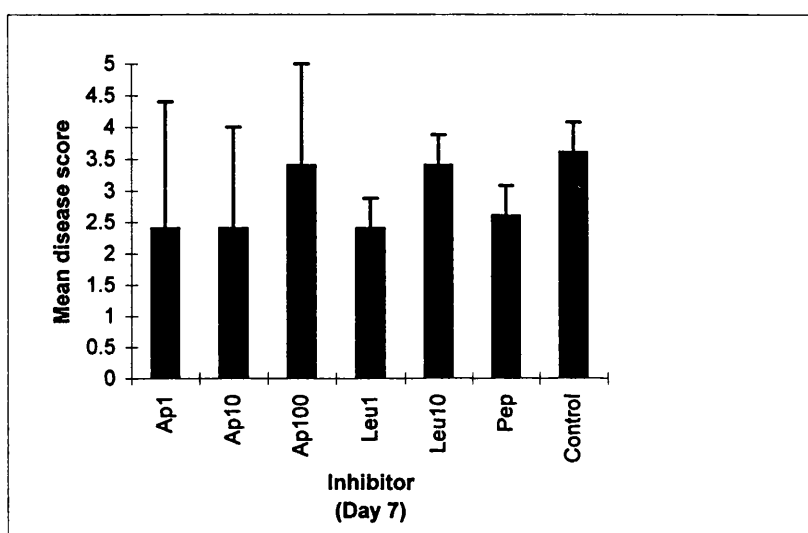
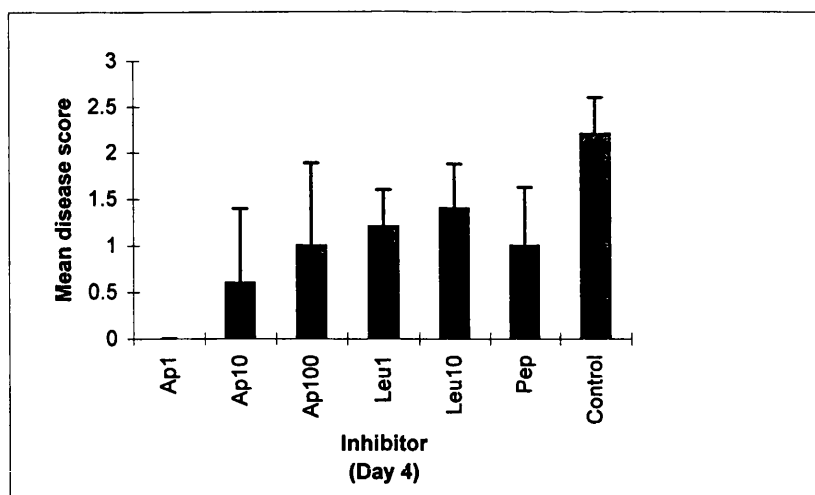


Fig. 4.34. Effect of commercial protease inhibitors on disease caused by *S.nodorum*. Each inhibitor was mixed with spores for 30min before inoculation. Symptoms were assessed at 4 and 7 days. A1=Aprotinin 1 μ M; A10=Aprotinin 10 μ M; A100=Aprotinin 100 μ M; Leu1=Leupeptin 1 μ M; Leu10=Leupeptin 10 μ M; Pep=Pepstatin 10 μ M; Con=Control. Disease score was: 0=no disease; 1=water soaked lesion; 2=chlorotic lesion; 3=necrotic spots within the chlorotic lesion; 4=necrotic lesion; 5=spreading necrotic lesion. Error bars represent the standard deviation about the mean of ten replicates.

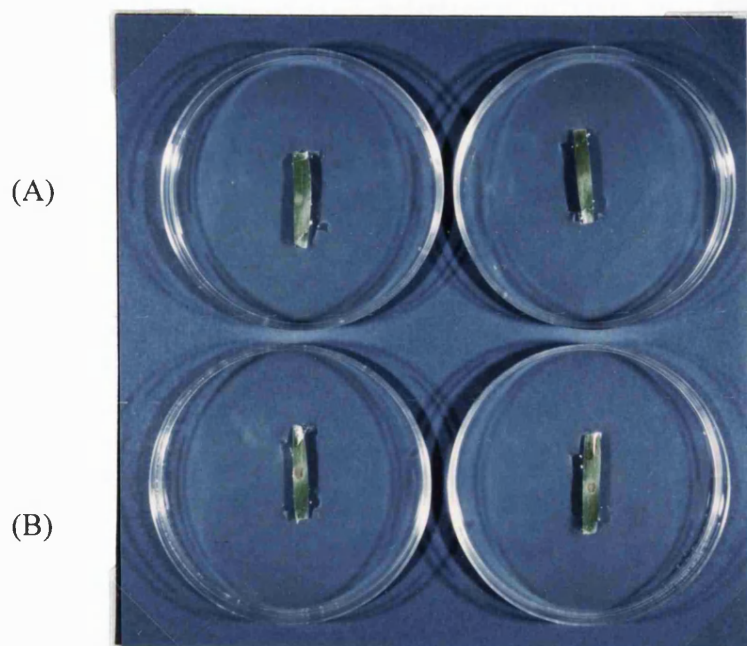


Fig. 4.35. Effect of aprotinin on disease caused by *S.nodorum*. Aprotinin ($1\mu\text{M}$ final concentration) was mixed with spores 30min before inoculation. (A) Control 6 days after inoculation; (B) $1\mu\text{M}$ Aprotinin 6 days after inoculation.

4.1.17.2 Effect of inhibitors on germination

The aim of this investigation was to determine whether the reduction in disease symptoms observed using protease inhibitors was due to inhibition of spore germination.

There was no inhibition of spore germination *in vitro* by these inhibitors; conversely the higher concentrations of inhibitors appeared slightly to stimulate germination over controls in distilled water Table 4.7.

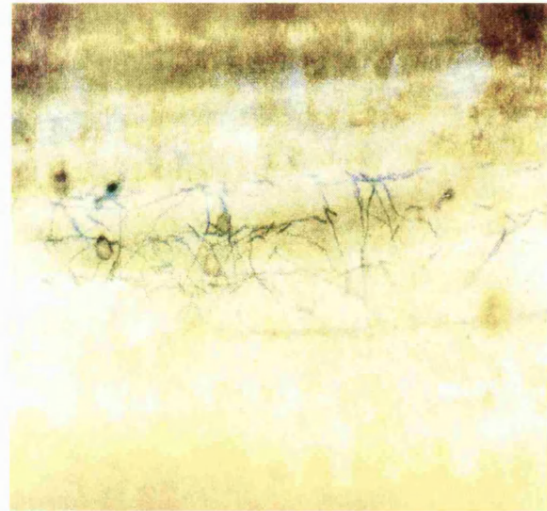
Treatment	Mean percentage germination (\pm SD)
Control	85 \pm 9.39
1 μ M Aprotinin	84 \pm 9.67
10 μ M Aprotinin	97.33 \pm 2.49
100 μ M Aprotinin	90 \pm 4.24
1mM Leupeptin	84.3 \pm 8.17
10mM Leupeptin	96.7 \pm 1.25
1 μ M Pepstatin	95.7 \pm 2.62

Table. 4.7. Percentage germination of spores mixed with protease inhibitors after 14h. Spores were mixed with protease inhibitors and allowed to germinate overnight on glass slides in a humid chamber at 20°C. After 14h percentage germination was scored for 100 spores in three fields of view for each treatment. The mean germination for each treatment and the standard deviation is shown above.

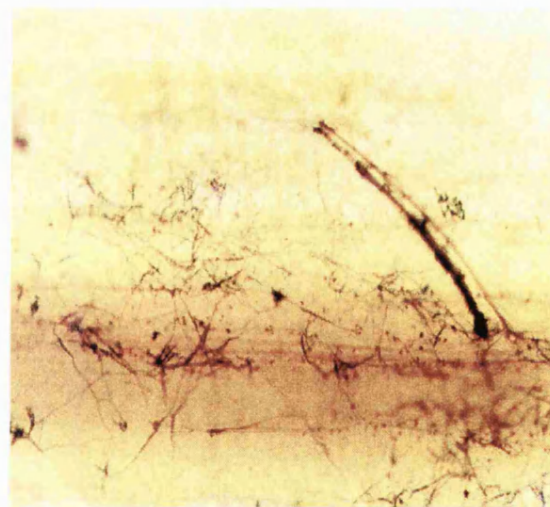
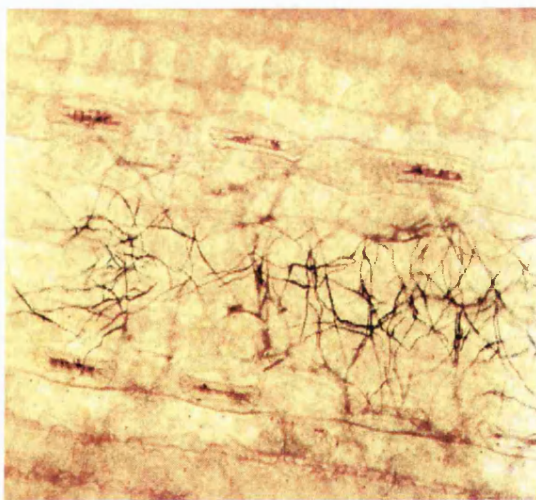
In contrast to the results *in vitro*, 100 μ M and 10 μ M aprotinin reduced germination on the leaf, while 1 μ M aprotinin and the other protease inhibitors did not. 100 μ M aprotinin prevented germination at 14h after inoculation (Fig.4.36) while 10 μ M aprotinin reduced germination by ca.50%. However, by 24h after inoculation, 100 μ M and 10 μ M aprotinin treated spores appeared no different from controls, with extensive hyphal growth across the leaf surface (Fig.4.36). Hence, the effect of higher aprotinin concentrations on germination appears to be transient and only displayed on the leaf surface. The fact that the most significant reduction in disease was not observed with these inhibitor concentrations indicates that this effect on germination does not influence disease development.

Aprotinin

Control



14h



24h

Fig.4.36. Effect of 100 μ M Aprotinin on the germination of *S.nodorum* spores 14 and 24h after inoculation. Aprotinin was mixed with spores for 30min prior to inoculation onto wheat leaves. Magnification (x200).

4.1.17.3 Effect of anti-SNP1 antibody on infection

The successful reduction of disease symptoms by defined protease inhibitors indicated a possible role for SNP1 in disease. However, this reduction in disease could not definitely be attributed to SNP1 inhibition and may have been due to the inhibition of an as yet uncharacterised trypsin activity induced *in planta*. To determine whether specific inhibition of SNP1 had an effect on disease, the antibody raised against SNP1 was mixed with spores, as previously described for the commercial inhibitors. First, inhibition of SNP1 activity *in vitro* was confirmed by mixing the antibody with pure SNP1 (300nkats) for 30min before assay (Table 4.8).

Antibody Treatment	% Activity (\pm SD)
Control	100
1:1	3 \pm 0.5
1:1/10	7 \pm 1.8
1: 1/100	71 \pm 1.3

Table 4.8. The effect of anti-SNP1 antibody on SNP1 activity as measured with the trypsin substrate Ben-Phe-Val-Arg-NA.

Undiluted and a 1/10 dilution of the antibody were effective at inhibiting almost all of the protease activity. A 1/100 dilution only reduced activity by 30%, consistent with the apparently low titre of this antibody (section 4.1.16).

Spores were mixed with undiluted antibody, 1:1, for 30min prior to inoculation onto the leaf. No significant differences in disease symptoms were observed at days 4 or 7 after inoculation compared to controls. No effect on germination, *in vitro* or on the leaf was detected.

The effect of infiltrating the anti-SNP1 antibody into the wheat leaf prior to inoculation with spores was also investigated, in collaboration with a final year project student, Adam Gapper. Leaves were infiltrated with three different

antibody concentration before inoculation with a range of spore concentrations from 10^3 to 10^7 spores per ml. Disease symptoms were assessed 7 days after inoculation (Fig.4.37; 4.38) and the data statistically analysed using the Mann-Whitney non-parametric test (Table. 4.9).

Spore conc	Antibody conc	P-value compared to control
10^3	0.1%	0.0232 *
	1.0%	0.048 *
	10%	0.0644
10^4	0.1%	0.0418 *
	1.0%	0.1368
	10%	0.0959
10^5	0.1%	0.0642
	1.0%	0.0399 *
	10%	0.0175 *
10^6	0.1%	0.0401 *
	1.0%	0.0016 **
	10%	0.0121 *
10^7	0.1%	0.4853
	1.0%	0.0354 *
	10%	0.0014 **

Table 4.9. Mann-Whitney (non-parametric) analysis of the effect of anti-SNP1 antibody infiltration into wheat leaves on disease symptoms caused by *S.nodorum* infection. Significance ($P<0.01$) is denoted by ** while ($P<0.05$) is denoted by *.

At 10^3 spores/ml significant control of disease was only obtained with 0.1% and 1% antibody concentration. Significant control was lost at the highest antibody concentration (10%), which is consistent with reduced control by the highest concentrations of aprotinin and leupeptin. A similar effect was observed with 10^4 spores/ml although in this case 1% antibody was ineffective as well as 10%. Higher concentrations of antibody, 1 and 10% were required to obtain significant disease control for 10^5 and 10^7 spores/ml. In contrast, all three antibody concentrations effectively reduced disease with 10^6 spores/ml.

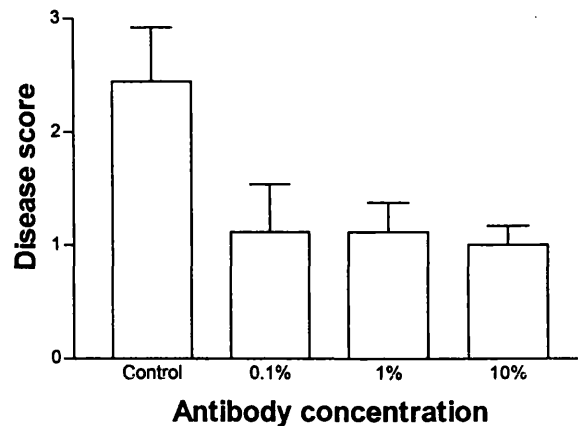
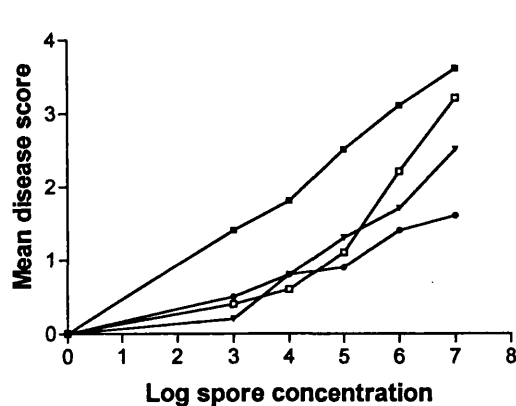
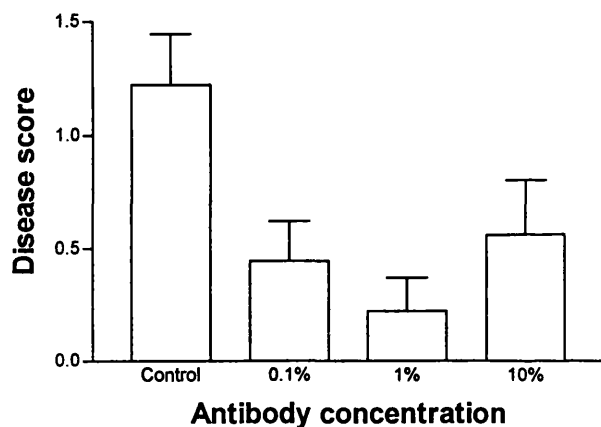
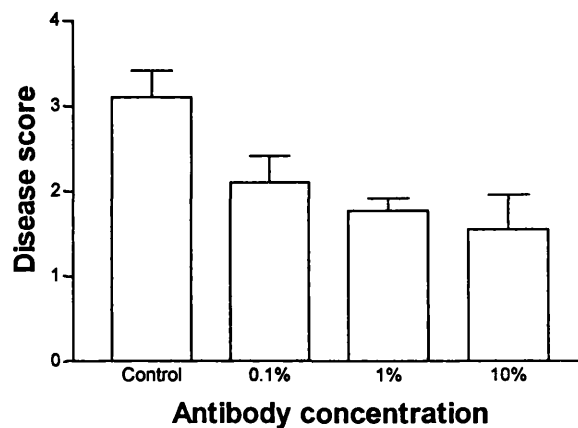


Fig. 4.37. Effect on disease score of infiltration of anti-SNP1 antibody into wheat leaves prior to inoculation of *S.nodorum* at a range of spore and antibody concentrations. Antibody concentrations were —●— 0, —□— 0.1%, —△— 1%, —◇— 10%. Detailed data with Standard deviations are shown in Fig.4.38 (a-e).

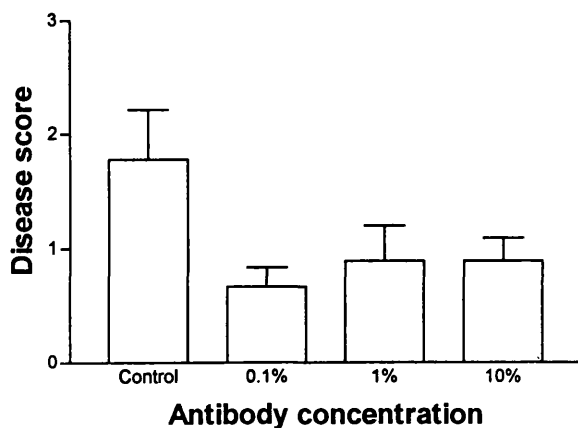
(c)



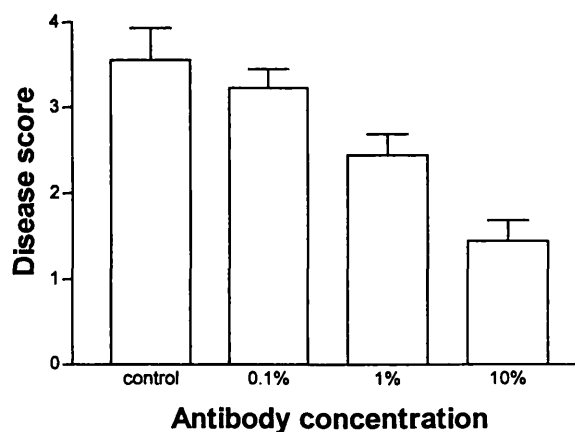
(a)



(d)



(b)



(e)

Fig.4.38. Effect on disease score of infiltration of anti-SNP1 antibody into wheat leaves prior to inoculation of *S.nodorum* at a range of spore concentrations (a) 10^3 ; (b) 10^4 ; (c) 10^5 ; (d) 10^6 ; (e) 10^7 spores/ml.

Overall, significant disease control was possible by antibody infiltration with generally higher spore concentrations requiring higher antibody concentrations for effective control.

4.1.18 Effect of SNP1 on tritordeum suspension cells

The ability of SNP1 to degrade the plant cell wall *in vitro* and its detection *in planta* during infection suggested that it may be destructive to plant cells. To test this, a range of SNP1 concentrations were tested against tritordeum suspension cells. Suspension cells were washed and resuspended to a final concentration of 0.05g/ml fresh weight in assay medium (0.5mM MES, 0.5mM CaCl₂, 0.5mM K₂SO₄ and 175mM mannitol and adjusted to pH5.7) prior to treatment with SNP1 or deionised water control. SNP1 was lyophilised and resuspended in assay medium at a range of concentrations and activity in this medium confirmed by enzyme assay. Concentrations used were, 0.04µg, 0.4µg, 4µg and 40µg of pure SNP1 protein, corresponding to 35nkats, 350nkats, 3.5µkats, 35 µkats of activity per ml of reaction respectively. Reactions were performed in triplicate and SNP1 incubated with suspension cells shaking at 120rpm at 25°C. 100µl of suspension was removed from each treatment at 2h, 6h, 24h and 48h and stained with the viability stain fluorescein diacetate (FDA). After 5min cells were examined using a fluorescence microscope and viability estimated based on the presence (viable) or absence (not-viable) of green fluorescent stain within the cells.

None of the treatments caused any significant death of the suspension cells under these conditions (Fig.4.39).

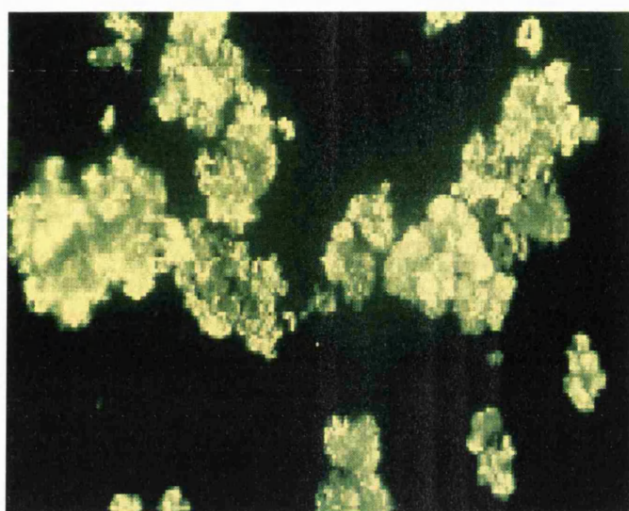


Fig.4.39. Tritordeum suspension cells following incubation with pure SNP1 for 48h and stained with the viability stain FDA. Magnification (x200).

4.2 Discussion

4.2.1 Characterisation of protease activity present in culture filtrate

S. nodorum produced at least two different classes of protease when grown on wheat cell walls. These have been classified as aspartic and trypsin-like proteases on the basis of inhibition characteristics and complete purification and sequencing of the trypsin-like protease, SNP1. In addition, none of the protease inhibitors were effective against the activity detected by azocasein at pH5, raising the possibility that at least one other protease is present that does not fall into one of the four general mechanistic classes. A number of proteases have been identified and isolated that do not fit the standard classification; these include the collagenases and the aminopeptidases (Neurath, 1989).

Only the use of an alternative protease assay using BSA as substrate at pH 3.2 enabled the aspartic protease to be detected. This activity was completely inhibited in the presence of pepstatin, an aspartic protease inhibitor that had no effect on the activity detected by azocasein. Hence, the azocasein substrate had failed to detect the aspartic protease. This may have been due to the assay pH of 5 which could have been too high; aspartic proteases typically function at low or neutral pH and are generally most active in the pH range 2-3 (North, 1982). However, some are active over a broader range, such as the aspartic protease from *Glomerella cingulata* (Clark *et al.*, 1997). Production of the *S. nodorum* aspartic protease at pH6 in liquid culture suggests that it is active at this pH and that the assay pH of 5 may not have been unsuitable. The pH optimum of this aspartic protease requires further investigation. If the pH was not limiting then the aspartic protease may not have been detected by azocasein because this was too insensitive or because the aspartic protease was unable to act on this substrate. The latter seems unlikely as many aspartic proteinases, such as rennin, exhibit milk-clotting activity, because they are able to cleave the Phe-Met bond of casein (North, 1982). Also, in a study by Lehtinen (1993), protease activities with low pIs were detected following isoelectric focusing by skimmed milk agar

overlay gels, indicating that if these are the same aspartic proteases they are active against casein.

The nature of the activity against azocasein at pH5 remains to be determined, particularly as no activity was detected by this method following Rotofor isoelectric focusing. This suggests that this activity may be unstable to extensive dialysis or isoelectric focusing; some enzymes are not active at their isoelectric point or activity may be lost due to ampholyte binding (An der Lan and Chrambach, 1987). The latter two problems can be tested by dialysis to an alternative pH with the prior addition of 1M NaCl which out-competes the ampholytes for sites on the protein. Rotofor isoelectric focusing did identify three different protease activities. One, a high pI activity of ca. 9.2 was detected by azocasein at pH8, while two aspartic protease forms were identified with the BSA assay at pH 3.2 with pIs of 2.6 and 4.5. The absence of activity in these fractions assayed by pH5 azocasein clearly demonstrated that the aspartic protease was not detected by this substrate. In contrast, gel isoelectric focusing followed by gelatin overlay only detected a single protease isoform, the high pI serine protease detected by Rotofor, with a pI of 8.7. No other protease activities were detected, including the aspartic proteases. These are either unable to degrade this substrate or the gelatin overlay is not sensitive enough to detect them if they are only produced at low levels.

Detection of protease activity in wheat cell wall culture filtrates contrasts with the work of Lehtinen (1993) which failed to detect any activity by assay. However in the same study activity was detected, as mentioned above, by skimmed milk agar overlay following gel isoelectric focusing. This method detected two protease activities with approximate pIs of 4 and 5, which may be the aspartic proteases detected in this study by Rotofor. In contrast to the work presented here, no high pI protease activity was detected using this overlay method. These discrepancies between studies clearly demonstrate the importance of using a range of substrates for both assay and overlay gels for the detection and characterisation of protease activity.

Aspartic proteases generally have isoelectric points below 5.1 and many contain carbohydrate, which is responsible for some proteinase heterogeneity (North, 1982). Therefore it is possible that the two aspartic proteases detected may result from differential glycosylation or processing rather than being distinct gene products. The aspartic proteases from the plant pathogenic fungi *Monilinia fructigena* (Hislop *et al.*, 1982) and *Botrytis cinerea* (Movahedi and Heale, 1990a) are both produced as single forms with a pI 4.0. Aspartic proteases have also been identified in a number of *Fusarium* species but not characterised (Urbanek and Yirdaw, 1978) and a single form has been purified from *Glomerella cingulata* (Clark *et al.*, 1997). These single forms contrast with the two forms identified by this work and the up to eight genes known to encode aspartic proteinases in *Candida albicans* (Monod *et al.*, 1994). The trypsin-like protease, SNP1, had a high pI of 8.7, similar to that of other fungal high pI proteases from *Alternaria teriussima*, *Fusarium sp.*, *Neurospora crassa* and *Tritirachium album* (North, 1982). This contrasts to a large number of serine proteases with more acidic pIs, such as the PR2 trypsins from *M.anisopliae* with pIs of 4.4 and 4.9 (St.Leger *et al.*, 1996a).

The production of different classes of proteases by the same fungus is common. The entomopathogen *M.anisopliae* produces trypsin-like, subtilisin-like and metallo-proteases as well as several exo-acting peptidases (St.Leger *et al.*, 1998). *C.carbonum* produces trypsin and subtilisin-like proteases (Murphy and Walton, 1996). The function of these different classes presumably reflects the substrates encountered by these fungi and perhaps their complementary roles *in vivo*. For example fungal aspartic proteases are known to be capable of activating trypsinogen (North, 1982).

4.2.2 Purification of an alkaline trypsin-like protease

An extracellular protease (SNP1) secreted by *S.nodorum* when grown on wheat cell walls as sole carbon source was purified to homogeneity by cation exchange chromatography. Inhibitor studies showed this to be a serine protease of the trypsin type, strongly inhibited by aprotinin and leupeptin and weakly by PMSF. PMSF reacts slowly with trypsins (Powers and Harper, 1986). N-terminal sequencing showed the enzyme to possess the conserved fungal trypsin N-terminus, with high homology to the other fungal plant pathogen trypsins of *Fusarium oxysporum* (Rypniewski *et al.*, 1993), *Cochliobolus carbonum* (Murphy and Walton, 1996) and *Verticillium dahliae* (Dobinson *et al.*, 1997).

The molecular weight of SNP1 of 25kDa is consistent with other fungal proteases (North, 1982), and is the same as Alp1a, a trypsin-like protease of *C.carbonum* (Murphy and Walton, 1996). The alkaline pH optimum of SNP1 is consistent with a number of other alkaline proteases from plant pathogens such as *C. cucumerinum* (Robertson, 1984), *C.lindemuthianum* (Ries and Albersheim, 1973), *U.maydis* (Hellmich and Schauz, 1988), *P.brassicae* (Ball *et al.* 1991) and the trypsin-like proteases of *C. carbonum* (Murphy and Walton, 1996), *F.oxysporum* (Rypniewski *et al.*, 1993) and *V.dahliae* (Dobinson *et al.*, 1997). SNP1 had a temperature optimum of 35°C at pH8 similar to the 40°C optimum observed for the trypsin-like protease of *F. oxysporum* (Rypniewski *et al.*, 1993). The activation of this enzyme by calcium ions is also consistent with it being a trypsin-like protease. A number of serine proteases have been shown to have a calcium ion requirement for thermal stability and resistance to degradation. It is also thought that this calcium requirement ensures that the enzymes are not active in the cytoplasm where the calcium concentration is low (Rypniewski *et al.* 1994). The calcium binding site in bovine trypsin is conserved in vertebrates but not in lower animals. In *Streptomyces griseus* it is located in a different position to that of bovine trypsin while in *S.erythraeus* (Yamane *et al.* 1991) and *F.oxysporum* (Rypniewski *et al.*, 1993) no calcium binding sites have been found. It is not possible to determine whether SNP1 has calcium binding sites

from the gene sequence as the sites are not conserved at the DNA level and their position is usually determined by X-ray crystallography (Rypniewski *et al.*, 1993). SNP1 does not have an absolute requirement for calcium, given that even very high concentrations of EDTA (100mM) only inhibited activity by 50% (data not shown). This is also consistent with trypsin-like proteases whose activity is merely stabilised by and not necessarily dependent on the presence of calcium (Salveson and Nagase, 1989).

The full length amino acid sequence of SNP1 indicates that it contains the conserved trypsin active site region CQGDSGGP and the catalytic triad His, Asp and Ser present in all trypsins (Rypniewski *et al.*, 1994). The asparagine lies at the bottom of the substrate binding site and its negative charge is responsible for binding the positively charged residues lysine and arginine in the substrate. The substrate specificity of SNP1 was typically trypsin-like, specifically cleaving the peptide bond on the carboxyl side of lysine or arginine residues (Kraut, 1977; Steitz and Shulman, 1982). The exact Arg/Lys preference for SNP1 requires further study with a range of other substrates identical in the P₂ and P₃ positions. Bovine trypsin shows a preference for lysine over arginine residues, while in *F.oxysporum* this specificity is reversed (Rypniewski *et al.*, 1993). Alterations in the P₂ and P₃ subsites also alter activity of SNP1; there was an 80% reduction in V_{max} between the Ben-Phe-Val-Arg-NA substrate and the Ben- Pro-Phe-Arg and a corresponding 3 fold increase in Km. This difference in activity was also observed for the trypsin-like proteases of the entomopathogenic fungus *Metarhizium anisopliae*, which showed a 92% reduction in V_{max} and a 10-fold increase in Km against the same substrates (St.Leger *et al.*, 1996a). When Phe is at the P₂ Lys was preferentially cleaved over Arg by SNP1, however this may also have been affected by a change from Pro to Val at the P₃. The ability of SNP1 to cleave lys residues may be of relevance in view of their abundance in the plant cell wall protein, extensin (Showalter, 1993). However, hydrolysis by trypsin is affected by the nature of the bonds, the side chains surrounding susceptible bonds and possibly by the surrounding sequence structure (Walsh and Wilcox, 1970). Hence the presence of lysyl bonds does not determine tryptic

susceptibility alone. Lysyl bonds flanked by polar side chains cleave slowly, and also, lysylproline peptide bonds are generally considered to be trypsin-resistant (Wilson and Yuan, 1989). Despite this, in a maize hydroxyproline-rich glycoprotein containing multiple Lys-Pro sequences one was tryptic labile while the other was stable (Kieliszewski *et al.*, 1989). For Lys-Pro cleavage, an extended polypeptide backbone (intrinsic to a proline-rich polypeptide) was necessary combined with backbone flexibility flanking the susceptible bond. Principally N-terminal flexibility was required at the -3 position and C-terminal flexibility at residue +2. Residues such as proline or hydroxyproline in these positions reduce flexibility and render the Lys-Pro bond resistant to trypsin. Whether SNP1 can cleave Lys-Pro bonds requires investigation in the context of extensin degradation.

4.2.3 Protease regulation

The regulation of SNP1 and the aspartic protease showed a similar pattern; induction of both proteases required the presence of a protein source. Both were highly induced by wheat cell walls, but while SNP1 was also highly induced by collagen, the aspartic protease was only weakly induced. However, the aspartic protease was more highly induced by the hydroxyproline containing proteins, wheat cell walls and collagen compared to BSA, gelatin and casein. The ability of protein sources other than wheat cell walls and collagen to induce SNP1 requires further investigation. It would also be interesting to see if peptides released from the wheat cell wall by either of the *S.nodorum* proteases are able to induce the other and any possible synergism that exists between the two. It has been suggested that peptides released by the basal levels of the trypsin-like protease, PR2 and the chymoelastase, PR1 of *M.anisopliae* induce PR1 still further (Paterson *et al.*, 1994).

It may be of relevance that the greatest induction of both SNP1 and the aspartic protease was by wheat cell walls. This contrasts to the trypsin-like proteases of the plant pathogenic fungus *C.carbonum*, which were not considered essential for

pathogenicity following gene disruption. These were equally induced by maize cell walls, gelatin, BSA, and casein but maximally by collagen (Murphy and Walton, 1996).

Both proteases were partially repressed by glucose and by nitrate and completely in the presence of both. SNP1 showed an equal level of repression by ammonium and nitrate which is interesting given that fungal proteases are generally more sensitive to repression by ammonium because this nitrogen source is usually preferentially utilised over nitrate (Marzluf, 1993). It may be that insufficient ammonium was included to observe any significant repressive effect. If so this may explain the complete lack of any repression by ammonium on the aspartic protease activity. In fact the presence of ammonium appeared to induce activity.

The regulation of both *S.nodorum* proteases appears to be controlled by carbon and nitrogen metabolite repression but there is also a requirement for a protein inducer, as seen in *U. maydis* (Hellmich and Schauz, 1988) and *N. crassa* (Cohen *et al.*, 1975). This is in contrast to *Aspergillus nidulans* where protease is induced by nitrogen, carbon or sulphur limitation and does not require the presence of protein (Cohen, 1981). Complete repression of both SNP1 and the aspartic protease appears to require the presence of both nitrogen and carbon. This co-operative repression is not uncommon. In *N.crassa* protease production is induced in the presence of protein and in the absence of either carbon, nitrogen or sulphur, and maximum repression is observed in the presence of all three (Cohen *et al.*, 1975). Other genes in filamentous fungi show a similar regulation, such as those required for catabolism of dual carbon and nitrogen sources, e.g. proline utilisation in *A.nidulans*, repression of which only occurs when both a repressing carbon and nitrogen source are present (Sohianopoulou *et al.*, 1993).

The efficient utilisation of growth substrates by filamentous fungi is under the control of both wide-domain and pathway specific regulatory genes (Marzluf, 1993). Particularly well studied are the carbon-catabolite and nitrogen-

metabolite repression mechanisms, primarily in the non-pathogenic fungi *Aspergillus nidulans* and *Neurospora crassa*. Similar wide-domain mechanisms have been identified in *Penicillium chrysogenum* (Haas *et al.*, 1995), *Trichoderma reesei*, *T.harzianum* (Ilmen *et al.*, 1996), *Magnaporthe grisea* (Lau and Hamer, 1996) and *M.anisopliae* (Screen *et al.*, 1997) suggesting they may be common amongst fungi. Carbon catabolite repression involves direct negative control of target promoters by a DNA-binding repressor protein such as Mig 1 from yeast or the closely related CREA repressor in *Aspergillus* (Ronne, 1995). Nitrogen regulation is mediated instead by positive acting regulatory proteins such as AREA and NIT2 from *A.nidulans* and *N.crassa* respectively (Marzluf,1993). Induction of specific pathways may still require a specific inducer, such as nitrate, which is required for the induction of synthesis of enzymes for nitrate assimilation. The nature of these regulatory proteins and their DNA binding sequences will be discussed in Section 4.3.4.

Whether these metabolic control circuits are important for pathogenicity is unknown, however nutritional signalling may play a role. For example, the *hrp* gene cluster of the bacterial plant pathogen *Erwinia amylovora* is required for both the pathogenic interaction with host plants and for induction of the hypersensitive response in resistant host and non-host plants (Wei *et al.*, 1992). Expression of these genes is repressed by high concentrations of ammonium ions and activated under nitrogen limitation. Similarly the *MPG1* gene in *M.grisea* which encodes a hydrophobin, essential for pathogenicity, is expressed under conditions of nitrogen or carbon limitation (Talbot *et al.*, 1993). To test whether loss of a similar global nitrogen regulatory gene, such as *nit2* or *areA* would affect pathogenicity, a major nitrogen regulatory gene was cloned and disrupted in *M.grisea* (Froeliger and Carpenter, 1996). The loss of this positive acting regulatory element, *NUT1* failed to have any effect on pathogenicity. Loss of this gene prevented growth on a range of nitrogen sources but the mutant was able to utilise other nitrogen compounds such as glutamate, proline and alanine, in contrast to *A.nidulans* mutants which are only able to utilise ammonium and glutamate. These results suggest that *M.grisea* may possess other nitrogen

control systems, functionally independent from *Nut1*, that may be more important to pathogenicity. Indeed two such genes *NPR1* and *NPR2* have been found in *M.grisea* (Lau and Hamer, 1996). Loss of these nitrogen pathogenicity regulation genes resulted in an inability to use a range of nitrogen sources and in a loss of pathogenicity. Both *NPR1* and *NPR2* are required for *MPG1* expression; mutants fail to show an increase in transcript of this pathogenicity gene during nitrogen starvation. Interestingly, *NPR2* mutants are also defective in appressorium formation. Which other pathogenicity genes in *M.grisea* may be affected by these mutations is unknown. The presence of an alternative nitrogen regulatory pathway involved in the regulation of certain pathogenicity genes requires investigation in other fungi.

The lack of repression by ammonia on the aspartic protease suggests that it is not regulated by the *areA/nit2* pathway, if indeed such a pathway exists in *S.nodorum*. Since the production of this protease is definitely regulated, there is presumably an alternative or additional nitrogen regulatory pathway in *S.nodorum* that may possibly be analogous to the *NPR1/2* pathway in *M.grisea*.

The range and type of proteases produced by fungi can also be regulated by pH. In *Aspergillus nidulans* acid phosphatases and proteases are secreted only in acidic environments and alkaline phosphatase is secreted only in alkaline environments (Caddick *et al.*, 1986). Six regulatory genes have been identified including the major zinc finger transcription factor PacC, an activator for alkaline-expressed genes and a repressor for acid-expressed genes (MacCabe *et al.*, 1996). The production of a range of proteases by *M.anisopliae* has been studied in a range of pH growth conditions. Proteases were only produced at ambient pHs in which they function effectively, irrespective of whether the medium contained an inducing cuticle substrate. The use of Northern analysis showed that the pH of the medium played a major role in gene expression, such that two subtilisin genes and the trypsin PR2 gene were turned off under acidic conditions. The detection of aspartic proteases, not produced *in vivo* due to the alkaline pH of infected cuticle, suggests that pH represents a physiological signal

that triggers the production of virulence factors (St.Leger *et al.*, 1998). The production of both a trypsin-like protease and aspartic proteases by *S.nodorum* at pH6 in liquid culture suggests that both of these enzymes are active at this pH. The pH of wheat infected by *S.nodorum* is unknown, although pH 5.9 has been determined in a previously study (Magro, 1984). If this is an accurate, representative measurement then both proteases should be present in infected tissue.

4.2.4 Isolation of *SNP1*

Heterologous library screening with the *try1* gene from *M.anisopliae* failed to identify the *SNP1* gene. This may have been because of the low stringency used or the presence of two introns in the *M.anisopliae* gene.

Degenerate PCR was successful in isolating most of the *SNP1* coding sequence, particularly once the N-terminal protein sequence had been obtained. Analysis of the *SNP1* PCR product confirmed that this was a typical trypsin-like protease, and as well as the conserved trypsin N-terminus contained the conserved trypsin active site region and conserved serine residue. This PCR product, used as a probe allowed successful isolation of the full gene encoding *SNP1* from a genomic library. Analysis of the gene sequence indicates that *SNP1* may be synthesised as a preproenzyme, consisting of a signal peptide, a propeptide and the mature protein. Many proteinases are synthesised in this way, including the trypsin-like proteases of *M.anisopliae* (Smithson *et al.*, 1995) and *C.carbonum* (Murphy and Walton, 1996) and several functions for the propeptide have been proposed (Baker *et al.*, 1993). These include the requirement of the propeptide for proper folding or secretion of the mature proteinase, anchoring the proteinase to the membrane and/or maintaining the proteinase in an inactive state until it is released from the cell. It is possible for the propeptide to perform one or more of these roles (Baker *et al.*, 1993). The 227-amino acid propeptide from the metalloproteinase from *A.fumigatus* has been shown to inhibit specifically the mature metalloenzyme (Markaryan *et al.*, 1996). This suggests that its role is to

keep the enzyme inactive until secretion. It remains to be seen whether shorter propeptides such as the 16aa one from *SNP1* perform a similar role.

The intron position in *SNP1* is conserved in the trypsin encoding genes *Alp1* of *C.carbonum* (Murphy and Walton, 1996) and *try1* of *M.anisopliae* (Smithson *et al.*, 1995), although the latter contained two introns. The intron position for *F.oxysporum* is unknown as the sequence available is a cDNA. The *SNP1* intron size of 56bp was consistent with the size of most fungal introns of less than 100bp (Gurr *et al.*, 1987) and the intron sizes of 74 and 90bp for *C.carbonum* and *M.anisopliae* respectively. A putative lariat site CCAACTT was identified in the *SNP1* intron showing high similarity to consensus lariat formation site SMRASNY (Ramsbosek and Leach, 1987). A similar sequence was found in the intron of *try1* from *M.anisopliae* (Smithson *et al.*, 1995).

Sequences typical of promoters of lower eukaryotes, TATAA and CAAC (Gurr *et al.*, 1987) were also detected. The function of these elements is unknown and they are not found in all fungal promoters (Gurr *et al.*, 1987). The more common sequence found is CAAT but two CAAC sequences were found in *SNP1* and one was found upstream of the trypsin-encoding gene, *Alp1* of *C.carbonum* (Murphy and Walton, 1996).

Analysis of the 5' upstream region of *SNP1* for regulatory sequences identified five putative CREA binding sites. The DNA binding zinc finger protein, CREA, of *A.nidulans* is known to bind to the consensus sequence 5' SYGGRG to effect carbon catabolite repression (Kulmburg *et al.*, 1993). The presence of a CREA-binding site is consistent with the glucose repression observed in liquid cultures. However, the consensus CREA-binding site CCGGGG and some others have been shown to be context dependent and only bind CREA when preceded by adenine at -2 and thymine at -1 (Espeso and Penalva, 1994). The putative CREA-binding sites in *SNP1* were not found in this context, suggesting that carbon-catabolite repression may be mediated by an alternative carbon-response protein. This has also been observed for the *egl1* gene of *T.reesei* and

T.longibrachiatum which are also C-regulated but CREA-binding sites were not in the correct context. However, it may be that the *Trichoderma* spp. and *Stagonospora* spp. CREA proteins may have a different binding-site specificity to that of *A.nidulans* CREA (Gonzalez *et al.*, 1994).

Eight putative AREA or NIT2 binding sites (GATA or the reverse, TATC) were identified in the 5' upstream region of *SNP1*. AREA and NIT2 from *A.nidulans* and *N.crassa* respectively are the most extensively characterised nitrogen regulatory elements, known to bind GATA sequence elements in either direction (Marzluf, 1993). Three forward and five reverse single GATA sites have been identified in the 5' upstream region of the *SNP1* gene. NIT2 shows highest binding affinity to sites which contain two (or more) GATA core sequences with spacing varying from 3 to 30bp (Marzluf, 1993). Single sites spaced further apart, as observed in *SNP1* only bind NIT2 weakly, which may explain the weak nitrogen repression observed for *SNP1* in liquid cultures. The inferred weak binding of nitrogen regulators and the presence of five poor consensus carbon regulatory binding sites suggests that *SNP1* may be regulated by alternative regulatory pathways, such as the *NPRI/2* pathway which regulates the pathogenicity gene *MPG1* in *M.grisea* (Lau and Hamer, 1996).

Also identified in the 5' upstream region were two cAMP responsive elements, consensus TGACGTCA (Lalli and Sassone-Corsi, 1994). One such element was identified in the promoter of the chymoelastase *prl* gene of the entomopathogenic fungus *Metarhizium anisopliae* (Screen *et al.*, 1997). There is evidence in this fungus that a cAMP-activated signal-transduction pathway regulates appressorium formation (St.Leger *et al.*, 1990). This is consistent with studies in the rice blast fungus, *Magnaporthe grisea* where cAMP has been shown to stimulate appressoria formation, even on non-inducing hydrophilic surfaces (Lee and Dean, 1993). Disruption of the gene encoding the catalytic subunit of a cAMP-dependent protein kinase (*CPKA*) in *M.grisea* only caused a delay in appressorium formation and appressorium production could still be stimulated by cAMP (Xu *et al.*, 1997). This evidence suggests that additional

cAMP-dependant protein kinase(s) are involved in surface sensing in *M.grisea*. Disruption mutants in the *CPKA* gene were non-pathogenic on rice unless inoculated through wounds, indicating that this gene is required for normal appressorial penetration and hence pathogenicity. In addition, a mitogen activated protein (MAP) kinase gene, *PMK1* (Pathogenicity MAP-Kinase 1) has been identified in *M.grisea* that is essential for appressoria formation that may act downstream of the cAMP signal transduction pathway (Xu and Hamer, 1996). cAMP may play roles in surface recognition, appressoria formation and appressorial penetration and hence in regulating pathogenicity. Whether cAMP regulates pathogenicity genes in other fungi, such as *S.nodorum* requires further investigation. If so, then *SNP1* could be one such gene.

4.2.5 Detection of SNP1 in planta

Despite the presence of a plant trypsin-like protease(s) it was possible to detect a trypsin-like activity in infected tissue not present in controls, that co-eluted from the S-column with SNP1. This activity was also completely inhibited by aprotinin and leupeptin which inhibited pure SNP1. Fractions co-eluting from day 2 extracts contained a 25kDa band not present in controls and those from day 14 contained a trypsin-like protease with a pI of 8.5 not present in controls. These results strongly suggested that the protease in these fractions was SNP1. Further characterisation of the protease activity in these fractions could investigate temperature and pH optima. The failure to detect SNP1 in co-eluting fractions from day 2 by gelatin overlay suggested that activity had been lost either during dialysis or from degradation by plant proteases; the SNP1 co-eluting peak was not resolved away from the plant trypsin-like protease. It was not possible to detect a 25kDa band distinct from controls on an SDS-PAGE of co-eluting fractions from day14, largely because of the abundance of a 31kDa band, not present in controls. This band may have been the 31kDa band detected in preliminary purification steps which was later identified as a xylanase (Chapter 5). SDS-PAGE of co-eluting day 7 fractions did not show a 25kDa band distinct from controls. Isoelectric focusing was not performed on day 7 extracts because

of the extremely low levels of co-eluting protease activity. The original extract from day 7 infected and control plant material contained ca. 40% and 80% less protease activity than that from days 2 and 14 respectively. Correspondingly, an 85% reduction in activity was observed in fractions co-eluting with the fungal trypsin compared to days 2 and 14. The reason for this lower activity is not clear, loss of activity during extraction was unlikely because all extracts were made at the same time using the same method. Losses may therefore be due to degradation by plant proteases or the presence of protease inhibitors. Alternatively, endogenous protease activity may be genuinely lower at this time point combined with a lower production of SNP1 as later observed by Western analysis.

A similar approach to detecting a fungal protease *in planta* was taken by Movahedi and Heale (1990b). Their work showed the co-elution by chromatography of an aspartic protease from infected carrot tissue with the aspartic protease from *Botrytis cinerea*. The co-eluting protease detected in infected tissue had a similar pI, pH optimum and sensitivity to inhibitors as the fungal proteinase. Another protease extracted from plants with similar elution and inhibitor characteristics to the fungal protease was detected in melon seedlings infected with *Colletotrichum lagenarium* (Playds *et al.*, 1981). This serine protease differed from the host protease which was inhibited by the cysteine protease inhibitor, iodoacetic acid. Aspartic proteases with similar pI and inhibitor characteristics to the pathogen have been detected in plants infected with *Fusarium culmorum*, *F.avenaceum* and *F.oxysporum* (Urbanek and Yirdaw, 1978). A high pI protease (9.9) was detected in bean infected with *Colletotrichum lindemuthianum* by column isoelectric focusing (Wijesundera *et al.*, 1989). While these approaches are valuable in giving an indication of presence *in planta* they are not definitive. A more common method for detecting protein expression *in planta* is by using specific antibodies, raised against the protein of interest.

Subsequent work using western blot analysis confirmed that SNP1 is produced by *S.nodorum* during infection of wheat. SNP1 was most clearly detected at later stages of infection, days 4 and 7, but was also detected 24h after inoculation. Only a very faint band was detected at day 2 suggesting that the reduced level of SNP1 observed at day 7 (different inoculation method) in the co-elution study was real.

Unfortunately the titre of the anti-SNP1 antibody was low; it was able to detect 1µg of pure SNP1 only down to a 1/1000 dilution (1h incubation) and higher concentrations of antibody, greater than 1/100 were required to inhibit SNP1 activity *in vitro*. It was only possible to use one rabbit for immunisation with SNP1 and this failed to produce high levels of specific antibody. It is well known that animals of the same species can differ in their immune responses, hence several animals are usually immunised at the same time in the hope that at least one of them may respond by producing a specific antiserum (Clausen, 1988). Fresh antibody to SNP1 should be raised by using several animals.

Because of the low titre of the antibody it was difficult to detect the presence of SNP1 *in planta* using western blot analysis and blots had to be incubated in a 1/10 dilution of antibody overnight. Despite the low titre of the antibody the level of SNP1 in infected tissue must have been low for such a lengthy incubation to be necessary. These low levels may be genuine or a result of proteolytic degradation by plant proteases. New antibody to SNP1 with a higher titre should be prepared and used to test whether SNP1 is present in such low abundance. If SNP1 is only produced at low levels *in planta* this does not lessen its potential importance in pathogenicity. The specific activity of the pure enzyme is high, typically 4.5mkats/mg. Hence low levels *in vivo* may be sufficient for its effect. Alternatively, only low levels may be detected if high levels of CWDE are produced in very localised regions such as during host penetration (Xu and Mendgen, 1997; St.Leger *et al.*, 1996a).

Detection of both SNP1 and the aspartic protease in cirrus mucilage confirmed the activity previously detected by azocasein, in Chapter 3. Investigation of

whether the SNP1 activity remains associated with spores following dilution and dispersal is now possible with the use of the antibody to SNP1.

Detection of SNP1 from early stages in infection is consistent with some other fungal proteases. Proteases have been detected associated with ungerminated spores (Fric and Wolf, 1994) and during the early stages of host infection by *B.cinerea* (Movahedi and Heale, 1990a) and *Metarhizium anisopliae* (St.Leger *et al.*, 1987). It is interesting that in infection of carrots by *B.cinerea* protease is the first enzyme to be detected, 10h before other wall degrading enzymes. Protease was also the first enzyme to be produced by *M.anisopliae*, during germination and appressoria formation on the host insect cuticle. The proteases of *Uromyces viciae-fabae* are developmentally regulated and are produced on artificial surfaces coincident with appressoria formation, after cellulase production but before pectinases (Deising *et al.*, 1995). These demonstrations of protease production in the very earliest stages of infection and the detection of SNP1 throughout infection suggests that they may play an important role in host colonisation.

4.2.6 Effect of standard protease inhibitors and anti-SNP1 antibody on disease symptoms

The involvement of SNP1 in disease was also supported by the observation that the trypsin inhibitors aprotinin and leupeptin slowed the progress of disease and that 1µM aprotinin at day 4 inhibited symptoms completely. Paradoxically, higher concentrations of aprotinin and leupeptin failed to inhibit infection and the higher concentrations of aprotinin slightly stimulated spore germination *in vitro*. This phenomenon may reflect the proteinaceous nature of these inhibitors which could have acted as a nutrient source. In contrast, 100µM and 10µM aprotinin inhibited germination *in vivo* by 100% and 50% respectively at 14h. This inhibition of germination contradicts with the effects observed *in vitro* suggesting that this effect of high aprotinin concentrations is only observed *in vivo*. The reason for this is unclear, although it may reflect some essential function of SNP1 during germination on the leaf that is not induced *in vitro*. Alternatively, there

may be some transformation of aprotinin once on the leaf surface that is inhibitory to spore germination. By 24h the effect of aprotinin on germination on the leaf was lost and growth appeared as extensive as controls. *S.nodorum* is known to make extensive growth across the leaf prior to penetration with only between 1-5% of penetration events being successful (Bird and Ride, 1981). If only a few successful penetrations are required this may explain why a transient inhibition of germination had no effect on subsequent pathogenicity. The lowest concentration of aprotinin and both concentrations of leupeptin had no effect on germination, but still reduced disease, implicating a role for SNP1 in disease. Pepstatin was also effective at day 4 in reducing disease symptoms with no effect on germination, suggesting that the aspartic protease also plays a role in infection. The addition of the commercial aspartic protease inhibitor, pepstatin, to *B.cinerea* spores prior to inoculation also reduced and in some cases completely inhibited infection (Movahedi and Heale, 1990b). This was with no apparent effect on germination.

By day 7 any reduction in disease symptoms had been lost, possibly because of degradation of the inhibitor by other proteases or simply that the fungus has grown out from the zone of influence of the inhibitor; *S.nodorum* often makes intensive and extensive mycelial growth on wheat leaves prior to penetration. Also penetration of leaves by *S.nodorum* would prevent the inhibitor(s) from reaching their target. It would be worth testing the combined effect of pepstatin and leupeptin, or aprotinin to see whether increased disease control could be achieved by inhibition of the two characterised proteases.

Failure of the antibody to SNP1, when mixed with spores to prevent disease was surprising given the effectiveness of the commercial inhibitors. The low titre of the antibody may not have been sufficient to fully inhibit the SNP1 activity produced on the leaf. Alternatively, the crude serum will contain many other components in addition to the antibody, including lipids and proteins which may have stimulated the growth of the *S.nodorum* spores away from this source of inhibitor or the antibody may have been degraded by other proteases induced by the presence of excess protein. Finally, the commercial trypsin inhibitors that

successfully reduced disease may not have been inhibiting SNP1 but other uncharacterised trypsin-like proteases that may only be induced *in vivo*.

In contrast, infiltration of the antibody to SNP1 into leaves reduced disease symptoms at day 7, indicating that the antibody is effective, but not during topical application. This demonstrates that SNP1 is required after penetration of the leaf, which could not be determined by the topical application of commercial inhibitors. Pre-immune serum should have been used as a control in these experiments. Although this was not done, antibody raised against a xylanase from *S.nodorum* (Xyl1) failed to reduce disease when infiltrated into wheat leaves (Chapter 5). This suggests that the reduction in disease symptoms observed in this work is unlikely to be a non-specific effect caused by antibody infiltration. Significant reductions in disease were observed at different antibody concentrations depending on the spore concentration inoculated. Significant reduction in disease was lost for 10^3 at the highest antibody concentration, similarly significant reduction in disease was only observed with the higher antibody concentrations for 10^7 spores. The reduction in disease control by higher antibody concentrations observed with 10^3 spores perhaps explains the lack of disease control with the neat antibody mixed with spores. 10% antibody was effective at controlling disease at 10^6 spores (the concentration used for topical application), five times this amount of antibody was used for topical application, and this may have stimulated growth. Alternatively, if SNP1 is produced in greater amounts on the leaf surface than during intercellular growth the low titre antibody may only have been effective when introduced into the plant by infiltration.

The significant reductions in disease at all spore concentrations suggests that SNP1 may play an important role in pathogenicity, both during early infection stages as demonstrated by topical applications with commercial inhibitors and during host colonisation as demonstrated by antibody infiltration. This is also consistent with the detection of SNP1 throughout infection by chromatography and Western analysis. The reduction in disease by pepstatin also implicates a

role for the aspartic protease in disease, although it still remains to be detected *in vivo*.

The successful reduction in disease by infiltration of antibodies to SNP1 into wheat leaves raises the possibility that transgenic expression of these antibodies could be used to protect plants from infection by *S.nodorum*. This technology, often known as 'plantibody' expression, is aimed at expressing monoclonal antibodies in plants to provide disease control and is under investigation as a novel crop protection strategy (Schots *et al.*, 1992). Antibody genes are cloned from mouse hybridoma cell lines and expressed in transgenic plants as whole antibodies or active fragments of the antibody (Conrad and Fiedler, 1994). Correct assembly of complete antibodies in the cytoplasm requires efficient targeting to the secretory pathway. This has been achieved in *Nicotiana tabacum* where expression of a full sized antibody specific to Tobacco Mosaic Virus reduced necrotic lesion numbers by up to 70% (Voss *et al.*, 1995). Expression of single chain Fv antibodies (scFv), recombinant polypeptides composed of the heavy- and light-chain variable domains of an immunoglobulin fused together by a flexible linker still requires correct folding but they do not have to be assembled like complete antibodies. Constitutive expression of a scFv against artichoke mottled crinkle virus in *Nicotiana benthamiana* significantly reduced and delayed the appearance of viral symptoms (Tavladoraki *et al.*, 1993).

The successful use of protease inhibitors in reducing disease is not surprising given that plants are well documented as producing protease inhibitors as part of their defence response to pathogen attack by insects and plant pathogens (Ryan, 1990). Peng and Black (1976) found that levels of trypsin inhibitor increased more in resistant tomato varieties than in susceptible ones in response to infection by *Phytophthora infestans*. Ten different families of protease inhibitors have been identified in plants and seven of these inhibit the serine class of proteases (Ryan, 1990). This is likely to reflect the abundance of these proteases in insect guts but may also reflect the importance of this class of protease for plant pathogens. The effectiveness of plant protease inhibitors against insect

pests has been clearly demonstrated but effects on plant pathogens have not been extensively studied. Proteinase inhibitors isolated from healthy bean and tomato plants reduced the activity of proteinases from *Fusarium solani* (Mosolov *et al.*, 1976) and *C.lindemuthianum* (Mosolov *et al.*, 1979). More recently, a study of trypsin and chymotrypsin inhibitors from cabbage foliage has shown that these exhibit antifungal activity *in vitro* against two species of phytopathogenic fungi, that do not attack cabbage (Lorito *et al.*, 1994). The inhibitors suppressed spore germination and germ tube elongation and also caused leakage of the intracellular contents. The latter is thought to be due to the inhibition of chitin synthesis in the cell wall because trypsin has been shown to regulate chitin synthase by proteolytically converting the precursor zymogen into an active form (Machida and Saito, 1993). Interestingly this inhibitor failed to have any effect on a cabbage pathogen, *Alternaria brassicicola* and the commercial inhibitor soybean trypsin inhibitor failed to have any effect on all three pathogens. Hence there appears to be a specific reaction between the plant protease inhibitors and the fungi. The exact site of action and physiological effect of other plant produced protease inhibitors is unknown, although it has been suggested that the prevention of proteolysis would limit availability of amino acids for growth and multiplication of the pathogen (Ryan, 1990).

In light of the failure of the cabbage proteinase inhibitor to have an effect on a cabbage pathogen it is interesting to note that a Bowman-Birk type protease inhibitor is produced by barley during the incompatible interaction with *S.nodorum* (Stevens *et al.*, 1996). This trypsin inhibitor may inhibit SNP1 and could in part be responsible for the incompatible reaction. The production of this inhibitor in wheat infected by *S.nodorum* also requires investigation along with its ability to inhibit SNP1.

Now that the *SNP1* gene has been cloned, gene disruption can be used to test the importance of this protease in disease and compared to the inhibitor work. However, reduction in disease by the use of inhibitors has not always been supported by gene disruption. Anti-cutinase antibodies have been shown to

prevent infection of pea stems by *Fusarium solani* f.sp. *pisi* and infection of papaya fruits by *Colletotrichum gloeosporioides* when mixed with the spores of these fungi (Kolattukudy, 1985). In contrast, disruption of the cutinase gene of *F.solani* failed to reduce disease (Stahl and Schafer, 1992). However, a subsequent, more detailed analysis of disrupted cutinase mutants has shown some reduction in disease (Rogers *et al.*, 1994). This latter study investigated multiple spore levels and used microscopic examination of developing lesions. High levels of spore inoculum, as used by Stahl and Schafer (1992) would have failed to detect any differences in virulence and only an end point examination of symptoms was assessed. Such high inoculum levels mean that disease progresses quickly and any differences in virulence would not be detected. The work with commercial inhibitors presented here supports the importance of early assessment of disease symptoms, while the antibody infiltration study demonstrates the differences in spore inoculum levels and their interaction with inhibitors. When disruption of SNP1 is achieved, careful studies will need to be made in order to truly determine its effect on pathogenicity. Circumstantial evidence at the current time strongly implicates a role for SNP1 in disease.

Such differences in methods of assessment of pathogenicity following gene disruption may explain the contradictory evidence for the importance of proteases in disease. Alternatively these may merely represent different strategies employed by different pathogens. Such a detailed study was performed following disruption of the *Alp1* trypsin encoding-gene from *C.carbonum* and no effect on pathogenicity was detected (Murphy and Walton, 1996). However, 55-65% of overall protease activity remained, partly attributed to the presence of a subtilisin-like protease. As well as realistic spore concentrations, realistic inoculation conditions are also required in order for sensible pathogenicity estimates to be made. Mutants of *Xanthomonas campestris* pv. *campestris* deficient in protease activity were shown to be unaltered in virulence on turnip when infiltrated into mature turnip leaves or seedlings (Tang *et al.*, 1987). However, in natural black rot infection, bacteria enter leaf veins through openings in the leaf margin called hydathodes. When protease-deficient mutants were introduced into mature turnip

leaves through cut vein endings rather than by direct introduction into the leaf intercellular spaces, a significant reduction in disease compared to wild type was observed (Dow *et al.*, 1990).

4.2.7 Degradation of cell wall proteins by SNP1

Detection of SNP1 *in planta* combined with reduction in disease as a result of its inhibition suggested that SNP1 has a role to play during infection of the plant. If SNP1 is a cell wall degrading enzyme then it would be expected to degrade wall proteins such as extensin, a hydroxy-proline rich glycoprotein. It is interesting therefore that SNP1 was produced in large amounts when grown on collagen in liquid culture, perhaps indicating that it can degrade this hydroxyproline-rich glycoprotein. However, induction by collagen does not necessarily indicate an *in vivo* ability to degrade the plant cell wall (St.Leger *et al.*, 1997).

Further confirmation of the possible role of SNP1 in cell wall degradation was obtained by the observation that pure SNP1 can release hydroxyproline-containing proteins from the wheat cell wall. No other enzyme activities were associated with pure SNP1 which clearly demonstrates that SNP1 is capable of degrading the plant cell wall alone. The release of wall fragments containing hydroxyproline suggests that these fragments may be released from hydroxyproline-containing plant cell wall proteins such as the extensins. Extensins in the walls of dicotyledons contain a characteristic repeated motif of Ser(Hyp)₄, in contrast the extensins of monocots are characteristically rich in threonine and proline, in addition to hydroxyproline, lysine and serine and contain alternative characteristic motifs, with only one “signature” Ser-(Hyp)₄ sequence. Interestingly in maize and sorghum HRGPs the majority of repeats contain a Lys substitution for a Hyp (Carpita, 1996) which may make them more susceptible to tryptic digestion. There are also distinct differences in the glycosylation of hydroxyproline residues; in dicotyledons most of the hydroxyproline residues are glycosylated with one to four arabinosyl residues

while in monocot extensin typically only half the hydroxyproline residues are glycosylated (Showalter, 1993). Characterisation of the glycosylation patterns of hydroxyproline in a range of plant species has shown that typically less than 30% of total hydroxyproline in the walls of monocots is glycosylated compared to greater than 80% in dicotyledons (Lamport and Miller, 1971). Digestion of extensin by trypsin has been used to study extensin amino acid sequences, however this usually has to be preceded by deglycosylation with anhydrous hydrogen fluoride (Smith *et al.*, 1986). The low glycosylation of, for example maize extensins, which is only 7-9% (Murphy and Hood, 1993) suggests that these monocot extensins may be more susceptible to degradation by proteases. Trypsin can cleave a hydroxyproline rich glycoprotein (HRGP) from maize (Kieliszewski *et al.*, 1989) but its ability to do so without prior deglycosylation has not been tested. Pathogens of dicots may require arabinosidases to act synergistically with proteases to achieve extensin degradation. Low levels of glycosylation in monocot HRGPs suggests that proteases may be able to degrade them directly. This has been demonstrated by this work, suggesting that SNP1 may play a role in wall degradation. It would be interesting now to test whether SNP1 can degrade the walls from other plant species, particularly dicots. The ability of SNP1 to degrade the wall may be of particular relevance given that an increase in wall extensin has been demonstrated in response to wounding and fungal attack and is thought to play a role in plant defence (Showalter, 1993). Enhanced deposition and cross linking of HRGPs is thought to play a role in increasing resistance of the host to the pathogen (Esquerre-Tugaye *et al.*, 1979). These proteins may present a physical barrier to the invading pathogen or they may immobilise pathogens by binding to them. A HRGP isolated from tobacco callus has been shown to be capable of agglutinate cells of an avirulent strain of the bacterial pathogen *Pseudomonas solanacearum* (Mellon and Helgeson, 1982). Interestingly this HRGP was unable to agglutinate cells of virulent isolates. Obviously degradation of these proteins would be advantageous to a plant pathogen.

In contrast to this work the Alp1a and Alp1b trypsin-like proteases of *C. carbonum* were produced on collagen but showed no activity against maize extensins. However, this work assessed degradation by SDS-PAGE and perhaps only low levels of degradation would not be detected by this method. Also, the study was on isolated maize extensin, whereas the work presented here on SNP1 only describes the release of wall fragments containing hydroxyproline residues. It may be that these are not from extensin and are from other hydroxyproline containing protein sources in the wall such as proline-rich proteins (PRPs) and arabinogalactan proteins (AGPs). PRPs are thought to be required for cross-linking of extensin precursors (Ye and Varner, 1991) and are also induced upon wounding much faster than extensin (Tierney *et al.*, 1988). Degradation of these proteins by SNP1 could also be very destructive.

Other isolated proteases have been tested against their host proteins. A metalloprotease produced by *Erwinia carotovora* spp. *carotovora* was able to degrade, *in vitro*, potato lectin, a hydroxyproline rich glycoprotein implicated in disease resistance (Heilbronn *et al.*, 1995). Similarly, an extracellular protease from another isolate of *E. carotovora* ssp. *carotovora* has been shown to degrade HRGPs from potato and tomato (Willis *et al.*, 1987). Recently another HRGP degrading protease has been isolated from the plant pathogen *Xanthomonas campestris* (Dow *et al.*, 1998). This metalloprotease was only detected due to its ability to degrade previously isolated HRGPs from turnip, *Brassica campestris*, and is different from three other proteases previously characterised based on β -casein substrate detection (Dow *et al.*, 1990). This new protease was not active against β -casein and the previously isolated proteases were not able to degrade the HRGPs. This raises the possibility that there may be proteases and other cell wall degrading enzymes as yet unidentified because of the absence of a suitable substrate. As the plant cell wall becomes better characterised so will the enzymes that degrade it. The work by Dow *et al.* (1998) also indicates that there may be proteases from dicot pathogens capable of degrading host HRGPs without prior deglycosylation. It is interesting that this protease was unable to degrade potato lectin, which contains long blocks of highly arabinosylated

contiguous Hyp residues in contrast to tomato extensin which it was able to degrade in which Ser-Hyp₄ groups are regularly interrupted by non-glycosylated primary amino acids.

It is also interesting to note that a high pI xylanase initially co-purified with SNP1 and this was one of the most abundant enzymes produced by *S.nodorum* when grown on wheat cell walls. The importance of xylan in the wheat cell wall (>40%) and the demonstration that this xylanase can also degrade the wall alone (see Chapter 5) suggests that these two enzymes may have a very destructive effect on the wall if secreted together *in vivo* and this requires further investigation.

Such a synergistic effect was observed between the aspartic protease and an endo-pectin lyase of *B.cinerea* on the hydrolysis of isolated carrot cell walls (Movahedi and Heale, 1990b). Pre-treatment of the walls with the protease resulted in a 40% increase in degradation subsequently by the endo-PL (Movahedi and Heale, 1990a). The aspartic protease was present in ungerminated spores and was the only enzyme detected in infected tissue up to 10h after inoculation, after which polygalacturonase, pectin lyase and pectin methyl esterase were detected. The detection of SNP1 early in infection may indicate that it plays a similar role.

4.2.8 Effect of SNP1 on tritordeum cells

The ability of SNP1 to degrade the wheat cell wall suggested that this enzyme may be destructive to the host during infection. However, SNP1 failed to kill tritordeum suspension cells after incubation with these cells. Tritordeum is a cross between wild barley and durum wheat (Barcelo *et al.*, 1993) and is known to be more resistant to infection by *S.nodorum* than wheat (Rubiales, *et al.*, 1996). Hence, there may be fundamental differences in tritordeum cell wall structure which *S.nodorum* CWDE may be unable to overcome. This should be tested by investigating whether SNP1 can degrade isolated tritordeum cell walls.

More specifically SNP1 should be tested against wheat cell suspensions, or wheat plants for example by infiltration into leaves and subsequent assessment of symptoms and cell viability. Cell suspensions are used instead of whole plants in order to identify more easily any effects of CWDE on plant cells. They are frequently used in studies of defence responses in plant-pathogen interactions (Peltonen *et al.*, 1997). Unfortunately no wheat cell suspension lines were available at the time of this work.

Alternatively, it may be that although the concentrations of SNP1 used in this work *in vitro* are considered extremely high they may represent only a small proportion of the activity generated by the pathogen *in vivo*. *In planta*, direct contact between fungal hyphae and the plant cell wall may allow very high concentrations of CWDE to be localised at their site of action, as was observed during the penetration of bean by *Uromyces vignae* (Xu and Mendgen, 1997).

The nature of this experiment really only allowed a detection of extensive cell death. This is a result of the nature of the suspension cells combined with the use of FDA as a viability stain. The suspension cells were generally found in small clumps of several cells and this may have made them more resistant to enzymic attack, but may also have prevented visual detection of the death of inner cells. It may be possible to sieve cells through a size filter in order to only obtain single cells or groups of only two or three cells. Any dead cells would then have been easier to identify. Also, the use of the fluorescent viability stain FDA may be complicated by naturally occurring fluorescent compounds in the cells giving false positives (Baker and Mock, 1994). The level of staining by FDA is also difficult to assess, some cells fluoresce more brightly than others and hence cell death is difficult to quantify. It may be possible to obtain a more accurate measurement of cell death with the additional use of the stain Evans blue. This stain can be used microscopically to stain dead cells but a spectrophotometric method allowing quantification of cell death has also been developed (Baker and Mock, 1994).

There have been few studies on the effects of proteases from plant pathogens on host cells, better studied have been the known tissue macerating enzymes such as pectate lyases and polygalacturonases (Bateman and Basham, 1976). The protease produced by *Erwinia carotovora* was unable to cause cell death in cucumber or potato, in contrast to endopolygalacturonate lyase which completely macerated and killed cucumber tissue within 1h (Tseng and Mount, 1974). The protease did however, cause cucumber protoplasts to burst, which the lyase did not. This suggests that in this case the protease may play a role in cell membrane damage rather than attacking the cell wall. In contrast, the aspartic protease of *B.cinerea* was unable to kill carrot protoplasts but was able to cause death of carrot suspension cells, suggesting that the presence of the cell wall was essential for phytotoxicity (Movahedi and Heale, 1990b). This protease also caused cell death in carrot root slices but it did not macerate the tissue. This protease, like SNP1, was able to degrade the host cell wall and had degraded 30% of protein in the wall after 2h. It is interesting therefore that this aspartic protease, able to degrade wall proteins killed suspension cells while SNP1 did not. An acid protease produced by *Monilinia fructigena* *in vitro* and in infected apple fruits failed to damage apple fruit tissue, cultured apple cells, plant protoplasts or release proteins from isolated fruit cell walls (Hislop *et al.*, 1982). This clearly demonstrates that the role of some proteases is not in cell wall or membrane degradation.

4.2.9 Other possible roles for proteases in pathogenicity

It has been suggested that the protease produced by *S.nodorum* may play a role in degrading the protein in the aleurone layer of seeds, when causing glume blotch (Sieber, 1989). The detection of SNP1 during infection of the whole plant suggests it also has a role to play during lesion formation on leaves. Its ability to degrade the wheat cell wall indicates that this role may be in host penetration and colonisation. However, SNP1 may also play a role obtaining nutrients, in countering host defence enzymes, in enzyme or toxin processing or it may be toxic *per se*. Detection of the aspartic protease of *S.nodorum* *in planta* has still

to be achieved, but this may play similar roles. Proteases are known to be involved in many physiological processes (Neurath, 1986) and roles have also been ascribed for them in the processing of enzymes, toxins and elicitors (North, 1982). The hole-forming toxin, aerolysin, produced by the bacteria *Aeromonas hydrophila* is processed to its active form by trypsin or its own protease (Howard and Buckley, 1985). Similarly the AVR9 race-specific elicitor of *Cladosporium fulvum* is processed first by fungal proteases and then by plant proteases to produce the stable active form (Van den Ackerveken *et al.*, 1993). The chitin synthase of the filamentous fungus *Absidia glauca* is also activated by treatment with trypsin (Machida and Saito, 1993). The importance of proteases in enzyme processing is demonstrated by pancreatic trypsin where it functions not only as a digestive enzyme but is also responsible for activating all the pancreatic enzymes, including itself by cleaving a short propeptide from the N-terminus of the inactive zymogen (Rypniewski *et al.*, 1994). In fact, many fungal aspartic proteases are also capable of activating pancreatic trypsinogen to trypsin, by cleaving at the same point as during autocatalysis (North, 1982). Both SNP1 and the aspartic protease may play roles in processing one another or other enzymes. Proteases are also thought to be responsible for generating multiple enzyme isoforms and a protease has been identified that is responsible for the *in vivo* and *in vitro* processing of a cellulase from *Streptomyces reticuli* (Moormann *et al.*, 1993). The proteolytic cleavage of an 82kDa cellulase produced a 42kDa enzyme and a 40kDa inactive protein. In addition to increasing the number of cellulase isoforms the action of this protease appeared to modulate the enzyme activity; while the 42kDa enzyme had the same substrate range as the 82kDa cellulase the 42kDa enzyme showed an increased activity against *p*-nitrophenyl- β -D-cellobioside (Schlochtermeyer *et al.*, 1992a). Sequence analysis has identified that cleavage of the 82kDa cellulase removes a putative cellulose-binding domain, hence a possible biological function of the protease may be the release of the enzyme from crystalline cellulose. This would result in a more efficient hydrolysis of soluble substrates like cello-oligomers generated from crystalline cellulose degradation (Schlochtermeyer *et al.*, 1992b). A pectate lyase, Pell from *Erwinia chrysanthemi* strain 3937 has also been shown to be processed

by its own extracellular proteases (Shevchik *et al.*, 1998). Processing did not alter *in vitro* activity of the pectate lyase but did increase its ability to macerate potato leaves; the smaller size and higher pI of the processed enzyme probably favoured its penetration into the plant cell wall. In addition, only the processed form was capable of eliciting the hypersensitive response in the non-host tobacco.

The aspartic proteinases of *Candida albicans*, in addition to their primary enzymatic role also enhance the adhesiveness of *C.albicans* to certain human tissues (Watts *et al.*, 1998). The exact mechanism is not understood but null mutants or cells in the presence of pepstatin show reduced adhesion. Proteases may also be required by plant pathogens to degrade host defence proteins such as chitinases and glucanases that are known to damage fungal cell walls (Mauch *et al.*, 1988). Finally, proteases may serve a purely nutritional role, as was claimed during the endophytic infection by *Acremonium typhinum* of its host grass (Lindstrom and Belanger, 1994).

The inhibitor studies and antibody infiltration experiments suggest that SNP1 has an important role to play during infection of wheat by *S.nodorum*. The fact that there are no reports of secretion of trypsin-like proteases by saprophytes (St.Leger *et al.*, 1997) suggests that these enzymes may have an important role to play in pathogenesis. This can now be tested by disruption of the SNP1 gene.

Chapter Five: Xylanases of *S.nodorum*

5.0 Introduction

β -1,4-Xylan is a heterogeneous polysaccharide found in plant cell walls which, after cellulose, is the second most abundant renewable polysaccharide in nature (Biely, 1985). β -1,4-Xylan is the major hemicellulose found in the primary wall of graminaceous monocots but is only a minor component of the primary wall of dicots (Brett and Waldron, 1996). The xylan found in monocotyledon primary walls is largely glucuronoarabinoxylan, in which arabinose is the dominant side chain. The arabinoxylans in the monocot primary wall are also substituted by ferulic acid, which may cross-link the arabinoxylans in the wall (Brett and Waldron, 1996).

Xylan degradation requires the synergistic action of endo- and exo-acting xylanases with accessory enzymes that remove side chain substituents from the xylan backbone, as described in Chapter 1 (Biely, 1985).

Xylanases are produced by a wide range of micro-organisms and their potential use in a number of commercial situations has led to considerable interest in them (Gilbert and Hazlewood, 1993). Possible commercial applications include: pre-treatment of forage crops to improve nutritional quality and digestibility of ruminant feeds; addition to pig and poultry cereal-based diets to improve nutrient utilisation; xylanase pre-treatment of paper pulps to remove xylan while maintaining cellulose content, and reducing dependence on chlorine in the brightening process; use of xylanase as a flour improver for bakery products. Because of these commercial applications xylanases have been studied in some detail in order to understand their structure and catalytic mechanism. It is hoped that such information will enable xylanase performance and efficiency to be improved for commercial use. The potential use of xylanases in digestion of waste products also illustrates the importance of these enzymes in carbon recycling by saprophytic micro-organisms.

Xylanases may also be important enzymes for pathogenic micro-organisms in overcoming the plant cell wall barrier. Xylanases are thought to be particularly important for pathogens of graminaceous monocots where up to 40% of the wall is arabinoxylan (Peltonen, 1995). Xylanase synthesis occurs early during infection of cereals by some pathogens and some pure xylanases can degrade isolated plant cell walls, supporting their possible role in infection (Cooper *et al.*, 1988; Baker *et al.*, 1977). However, gene disruption of several xylanases from the plant pathogens *M.grisea* and *C.carbonum* have failed to have any effect on pathogenicity (Wu *et al.*, 1997; Apel-Birkhold *et al.*, 1996). Although, in both these studies residual activity remained, due to the presence of previously unidentified xylanase isoforms.

Most micro-organisms that produce xylanases synthesise a range of isoenzymes with very similar but not identical substrate specificities (Wong *et al.*, 1988). Multiplicity presumably reflects the fact that not all xylosidic linkages are equivalent and not all are equally accessible to xylanolytic enzymes. Also, this accessibility will change during the course of xylan hydrolysis. For example an unusual sequence dependent xylanase has been found that only cleaves a β -1,4-xylosyl linkage in the presence of an appendant glucuronosyl unit (Nishitani and Nevins, 1991). Removal of glucuronosyl groups by α -glucuronidase during xylan hydrolysis may prevent this xylanase from working, hence its role may only be in the early stages of xylan degradation.

Xylanase multiplicity may derive from distinct gene products or may be apparent and arise from a number of other sources (Wong, 1988). These include substrate cross-specificity by other enzymes, such as cellulases; post-translational modifications, such as glycosylation and proteolysis; degeneration of xylanases by proteolysis.

Many xylanases do derive from distinct gene products and the isolation of xylanase genes has provided information about the primary structures of

xylanases. Amino acid sequence analysis, coupled with hydrophobic cluster analysis has enabled the evolutionary relationships of these xylanases and other glycosyl hydrolases to be determined (Henrissat and Bairoch, 1996). As a result 57 families, based on 950 sequences have been identified, 34 of which are polyspecific *ie* contain at least two EC numbers (Henrissat and Bairoch, 1996). Cellulases and xylanases were among the first types of glycosyl hydrolases to be classified in this way into 11 families A-K (Gilkes *et al.*, 1991). Xylanases were found in only two groups F and G, and these now correspond to groups 10 and 11. The latest database information shows that group 10 contains 23 xylanases while group 11 contains 17 from a range of micro-organisms, although one micro-organism may possess xylanases in both categories (Henrissat and Bairoch, 1996). All the xylanases in group 10 are high molecular weight with generally a low pI and consist of a cellulose-binding domain and a catalytic domain connected by a flexible linker region. The xylanases of group 11 are low molecular weight and highly specific and although a very homologous group, can be divided into alkaline and acidic pI xylanases (Torronen and Rouvinen, 1997). The most conserved region within each family appears to be the catalytic domain and the most differences are found in the N-terminus. No significant homology is found between the two families, in fact they belong to different clans. A 'clan' is a group of families that are thought to have a common ancestry and are recognised by significant similarities in tertiary structure together with conservation of the catalytic residues and catalytic mechanism. On this basis, group 10 belong to clan GH-A along with groups 1,2,5,17,30,35,39 and 42, while group 11 belong to clan GH-C along with group 12 (Henrissat and Bairoch, 1996). Despite these differences both xylanase groups act by the same catalytic mechanism. There are two major mechanisms for glycosyl hydrolases, retaining or inverting (McCarter and Withers, 1994). Both groups of xylanases act *via* a retaining mechanism and two conserved glutamate residues are the catalytically active residues located either side of the active site cleft. These act as a nucleophile and an acid/base catalyst (Torronen and Rouvinen, 1997). Another common feature of the two groups of xylanases is their endo mode of action which can be demonstrated viscometrically (Biely *et al.*, 1997). While

similarities exist between their mode of action there are differences in their catalytic versatility and hence substrate specificity. Group 10 xylanases have a lower substrate specificity than group 11 and family 10 xylanases require two unsubstituted xylopyranosyl residues between the branches, while family 11 requires three. The xylanases from family 10 are therefore better at cleaving glycosidic linkages in the xylan main chain closer to substituents such as glucuronic acid and acetic acid. Replacement of β -1,4-linkages by β -1,3-linkages also represents a more serious barrier to family 11 xylanases than for family 10 (Biely *et al.*, 1997). Hence, family 10 xylanases release smaller products than family 11 and family 10 xylanases can further hydrolyse the oligosaccharides released by family 11 xylanases. Family 10 xylanases as a rule can also catalyse hydrolysis of cellulase substrates, aryl β -D-cellobiosides at the agluconic linkage (Biely *et al.*, 1997). They also possess several catalytic activities which are compatible with the β -xylosidases and have a higher catalytic activity towards shorter xylooligosaccharides than family 11. Despite the larger size of family 10 xylanases they have a smaller substrate binding site than family 11. Their greater catalytic versatility can be ascribed to differences in their tertiary structure which has been established by crystallography. The family 11 xylanases are smaller, well packed molecules. Their active site is a deep cleft and this contrasts with only a shallow bowl in family 10. This difference, combined with the potentially greater conformational flexibility of the larger enzymes than of the smaller enzymes may account for a lower substrate specificity in family 10 (Biely *et al.*, 1997).

Endoxylanases generally conform to three basic types, (i) small (20-30kDa) single-domain enzymes; (ii) modular enzymes comprising multiple catalytic domains; (iii) modular enzymes consisting of a catalytic domain fused to a non-catalytic domain fused to a non-catalytic cellulose binding domain (Black *et al.*, 1994). Recent work has shown that cellulose-binding domains enhance the activity of hemicellulases against complex substrates, probably by increasing the effective enzyme concentration at the substrate surface (Black *et al.*, 1996). Other non-catalytic binding domains include thermo-stabilising domains,

docking domains for formation of aggregated enzyme complexes, and domains homologous with the NodB protein from nitrogen-fixing bacteria with possible deacetylation activity (Laurie *et al.*, 1997).

The production of multiple xylanases with different catalytic properties by many micro-organisms indicates that their synergistic action is required in achieving xylan degradation.

Preliminary studies *in vitro* and *in vivo* had detected high levels of xylanase production by *S.nodorum* (Chapter 3). The aim of this work was to characterise this xylanase activity further, to determine its complexity and confirm detection in infected tissue.

5.1 Results

5.1.1 Regulation of xylanase production by different carbon sources

Xylanase activity has been detected in cultures of *S.nodorum* grown on wheat cell walls (Chapter 3). To investigate the regulation of xylanase production, *S.nodorum* was grown on a range of other carbon sources. Established biomasses were starved and transferred to the different carbon sources and cultures sampled at 12, 24 and 36 hours. Xylanase production was greatest in cultures grown on wheat cell walls and was about twice that produced on oat spelt xylan (Fig.5.1). No xylanase was produced in cultures grown on glucose or on cell walls plus glucose. Some xylanase activity was detected in the latter culture at 36h, perhaps due to glucose depletion. Xylanase production equal to that on oat spelt xylan was also observed on xylose. Pectin was included as a non-inducing polymer, however some xylanase activity was produced.

5.1.2 Characterisation of the isoforms of xylanase produced by *S.nodorum* on wheat cell walls *in vitro*

Xylanase multiplicity is common in microbial xylanolytic systems (Sunna and Antranikian, 1997). The aim of this aspect of the work was to determine how many isoforms were produced by *S.nodorum* in liquid culture on 1% wheat cell walls. Two methods were employed to separate xylanase isoforms on the basis of their isoelectric points (pI), Rotofor TM preparative IEF and gel IEF.

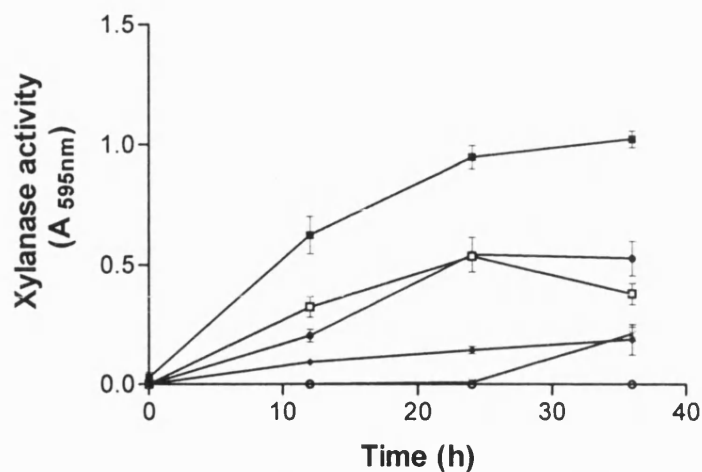


Fig.5.1. Carbon regulation of xylanase production in liquid cultures of *S.nodorum* grown on a range of carbon sources. Cell wall (■), Cell wall +glucose (X), Pectin (◆), Xylose (□), Oat spelt xylan (●) and Glucose (○). Error bars represent the standard deviation about the mean of three replicate flasks.

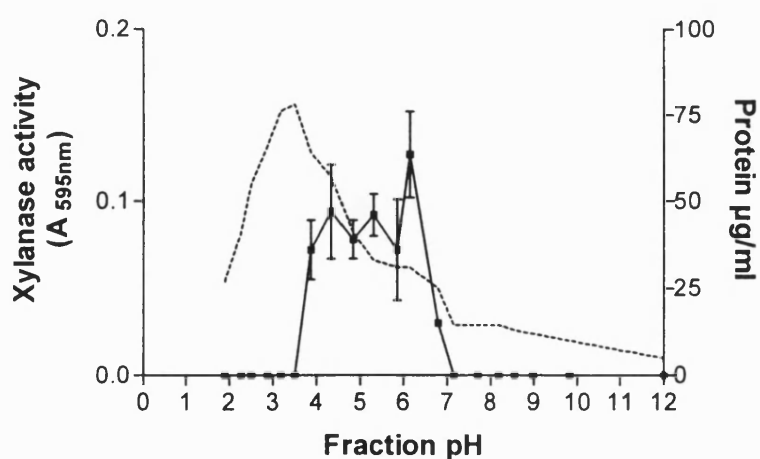


Fig.5.2. Rotofor separation of 48h culture filtrates from *S.nodorum* grown on 1% wheat cell walls. Xylanase activity (■) assayed with RBB-dyed xylan. Error bars represent the standard deviation about the mean of two independant separations.

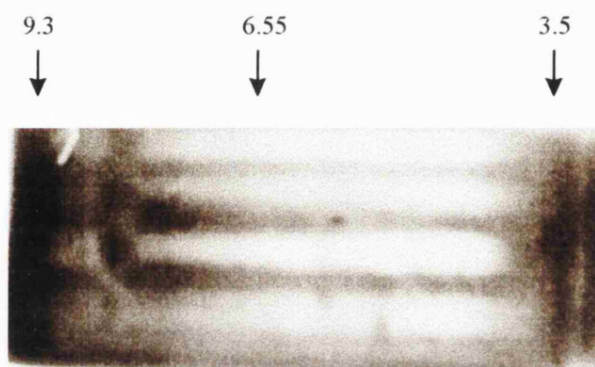


Fig.5.3. RBB-dyed xylan overlay gel detecting xylanase activity in fluids from cell wall grown cultures of *S.nodorum* following isoelectric focusing.

5.1.2.1 Rotofor™ Preparative Isoelectric focusing

Dialysed 48h cell wall grown culture filtrate was separated using Rotofor™ preparative isoelectric focusing in the pH range 3-10 (Fig. 5.2). Xylanase activity was assayed using RBB-dyed xylan because this substrate is specific for endo-acting xylanases. Xylanase activity was detected in all fractions from pH 3.8 to 6.8, suggesting that a number of low pI isoforms exist. Activity was similar in all of these fractions with the peak in activity at pH 6.2. No high pI activity was detected.

5.1.2.2 Gel isoelectric focusing

Culture filtrate proteins were separated in the pH range, 3.5-9.5 by electrofocusing in a pre-made IEF gel. Following separation, gels were overlaid with an RBB-dyed xylan activity gel. Unfortunately only a large clearing zone appeared spanning pIs 4-7 (Fig.5.3) rather than discrete bands of activity. This was in spite of using a range of protein concentrations (50, 100, 250 and 500µg) and temperatures (RT, 40, 50 °C) in attempts to generate distinct clearing zones. This problem suggested that a large number of isoforms with very similar pIs exist, consistent with the Rotofor™ results. Oat spelt xylan overlay gels were also tried, prepared as above, but using different methods for detecting clearing zones. Gels were washed in 95% ethanol (Royer and Nakas, 1990) or with 1M NaCl followed by staining with 0.1% Congo red (binds to β-1,4-glucans) for 30min followed by further washes in 1M NaCl (MacKenzie and Williams, 1984). The disadvantage of these other methods is that it is not possible to watch clearing zones appear before the gel is 'developed', unlike the RBB-dyed xylan overlay. These other methods were also unsuccessful.

While this isoelectric focusing work did not determine the number of xylanase isoforms present in culture filtrate it indicated that there were a large number with very similar pIs. The pH range over which clearing zones appeared was consistent with the Rotofor results, ranging from pH 4-7.

5.1.3 Identification of a high pI xylanase activity

Preliminary attempts to purify the trypsin-like protease using cation exchange chromatography as described in Chapter 4, generated peak fractions containing two major protein bands (Figs.5.4, 5.5). Further work using higher resolution separation techniques (Chapter 4) enabled the trypsin-like protease to be purified, confirming that this was the lower, 25kDa band seen in Fig.5.5. The upper 30kDa band appeared to be an extremely abundant protein in cell wall culture filtrates and this raised the possibility that it was highly induced by wheat cell walls. Because of this, attempts were made to determine if this was another cell wall degrading enzyme. Peak fractions were assayed for all the CWDE detected in liquid cultures in Chapter 3. The only activity found in these fractions, other than the trypsin, was xylanase (Fig.5.4), suggesting that this 30kDa band was a xylanase.

Because this xylanase activity had bound to an S-column at pH6 it was likely to be a high pI xylanase. This was unexpected because no high pI xylanase activity had been detected in the previous isoelectric focusing studies or in previous studies by Lehtinen (1993) and Magro (1984). To determine the pI of the partially purified xylanase two 20µg aliquots of fraction 10 were separated using gel isoelectric focusing. One aliquot was stained along with a pI marker and 200µg of crude culture filtrate while the other was overlaid with an RBB-dyed xylan containing gel (Fig. 5.6).

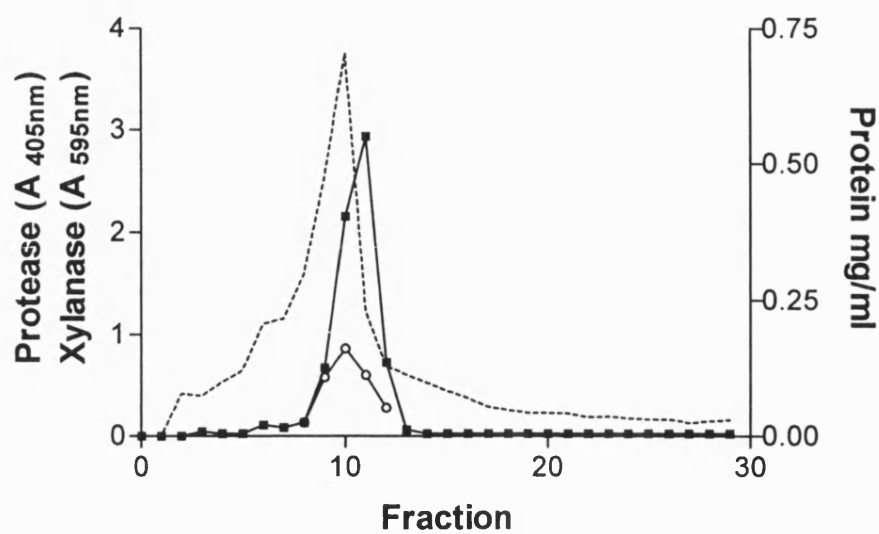


Fig. 5.4. Partial purification of SNP1 and a high pI xylanase using cation exchange chromatography. Protein mg/ml (----), Protease activity A_{405nm} (■), Xylanase activity A_{595nm} (○).

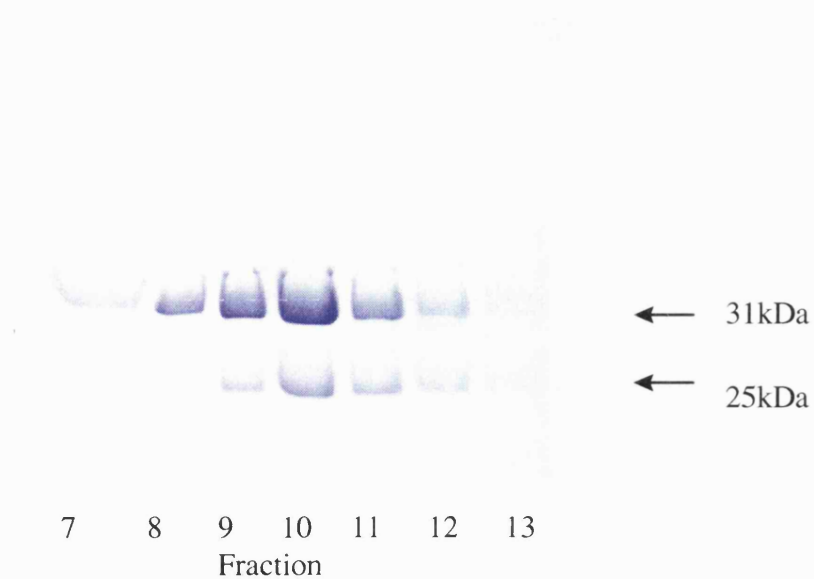


Fig.5.5. SDS-PAGE of S-column fractions containing protease and xylanase activity. Crude 48h *S.nodorum* cell wall grown culture filtrate was loaded onto a 10ml S-sepharose column in 25mM MES pH6.0 and eluted over a 0-250mM NaCl gradient (Fig.5.4).

Following incubation of the IEF gel in contact with the overlay gel a distinct clearing zone appeared corresponding to the more abundant protein from fraction 10. The other, fainter band, corresponded to the trypsin, pI 8.7. This confirmed that the 30kD band was indeed a xylanase with a high pI of greater than 9.3. Its exact isoelectric point cannot be determined because during isoelectric focusing the protein became focused to the edge of the gel at the basic end. The xylanase therefore has a pI of 10 or greater (the effective separation range of the gel is 3-10).

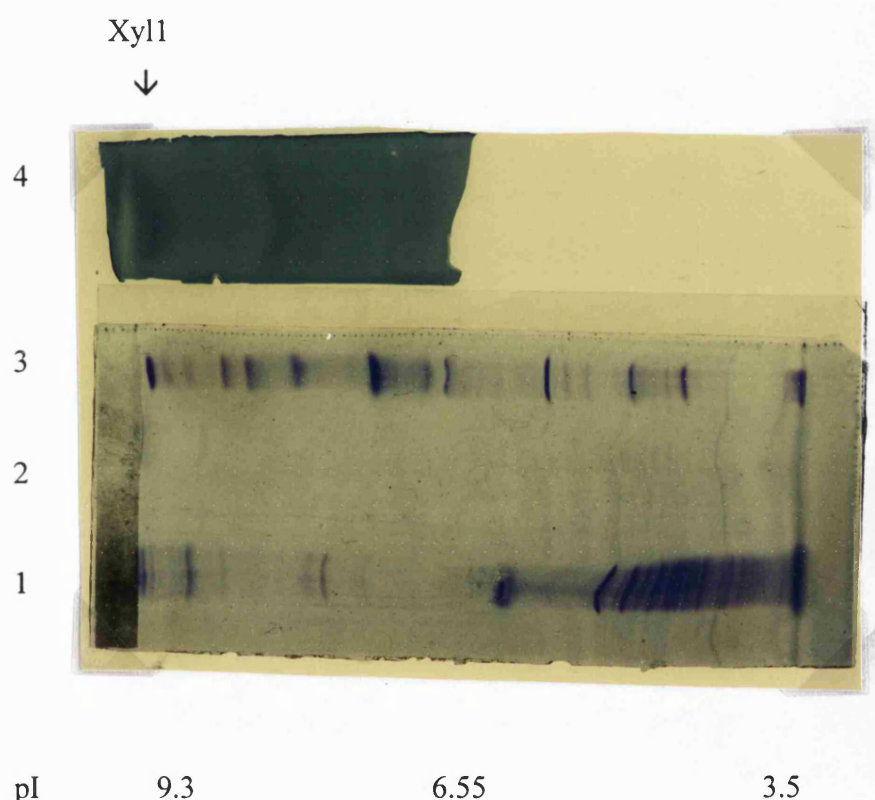


Fig.5.6. Isoelectric focusing gel showing in lane 1, 100 μ g of crude 48h cell wall grown culture filtrate; lane 2 partially purified xylanase, Xyl1 from S-Column fraction 10 (Fig.5.4); lane 3 pI marker, lane 4 RBB-dyed xylan overlay gel following incubation with lane 2 (Xyl1) of a similar IEF gel. Clearing zone indicated corresponds to xylanase activity.

This work clearly demonstrated that a high pI xylanase, secreted by *S.nodorum* on 1% wheat cell walls had been partially purified by a single step of cation exchange chromatography. Following this discovery further characterisation of the xylanase (Xyl1) was undertaken.

5.1.4 Temperature and pH optimum of Xyl1

The aim of this work was to determine the temperature and pH optimum of the partially purified xylanase (Xyl1) using the Nelson-Somogyi reducing sugar assay. The pH optimum was determined first by assaying over a pH range (2-11) using a series of overlapping buffers all at 50mM; Glycine-HCl pH 2.0-3.0, Citrate 3.0-7.0, MOPS pH7.0-8.0; Tris-HCl pH8.0-9.0; CAPS pH 9.0-11.(Fig.5.7). Xyl1 had a broad pH optimum over the range pH 5-9 with activity sharply declining either side of these pH values. Optimal activity was found at pH 7.0.

Xylanase activity was then assayed at pH 7.0 over a range of temperatures from 20-80 °C (Fig. 5.8). Xyl1 was active over all of these temperatures, but showed peak activity at 50°C. Xyl1 still showed approximately 20% of optimal activity even at 80°C, indicating that it is relatively heat stable.

5.1.5 Confirmation of endo-action

Xylanases can act either by an endo-action, cleaving within the xylan backbone or an exo-action removing single xylose groups from the end of the xylan molecule. The aim of this work was to confirm that Xyl1 was an endo-acting xylanase. It is not possible to determine whether a xylanase is endo-acting on the basis of activity in the Nelson-Somoygi assay. This is because the assay is non-specific and any glycohydrolase acting on the birch wood xylan substrate would be detected by this method including exo-acting xylanases. It was likely that Xyl1 was endo-acting because it cleaved the RBB-dyed xylan substrate, which is specific for endo-acting β -1,4 xylanases (Biely, 1985a). However, endo-acting enzymes are routinely detected using viscometric assays, where internal cleavage at several points along the polymer backbone by an endoacting enzyme should cause a rapid decrease in the viscosity of the xylan substrate. In contrast, exo-acting enzymes should act more slowly, as these can only remove single xylose groups from the end of the polymer, one at a time.

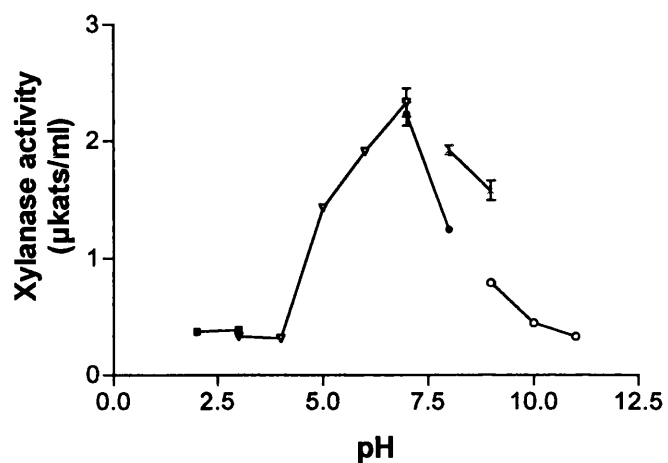


Fig. 5.7. pH optimum of Xyl1, determined using five overlapping buffers. Glycine-HCl (—■—) Citrate (—▼—), MOPS (—●—), Tris-HCl (—○—), CAPS (—○—). Error bars represent the standard deviation about the mean of three replicates.

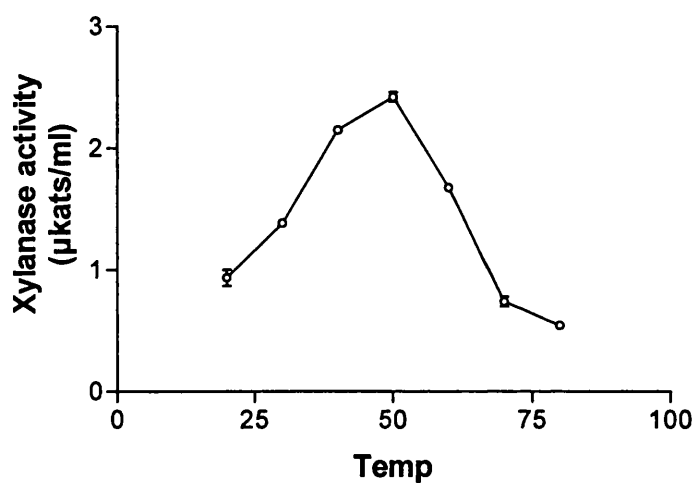


Fig. 5.8. Temperature optimum of Xyl1. Error bars represent the standard deviation about the mean of three replicates.

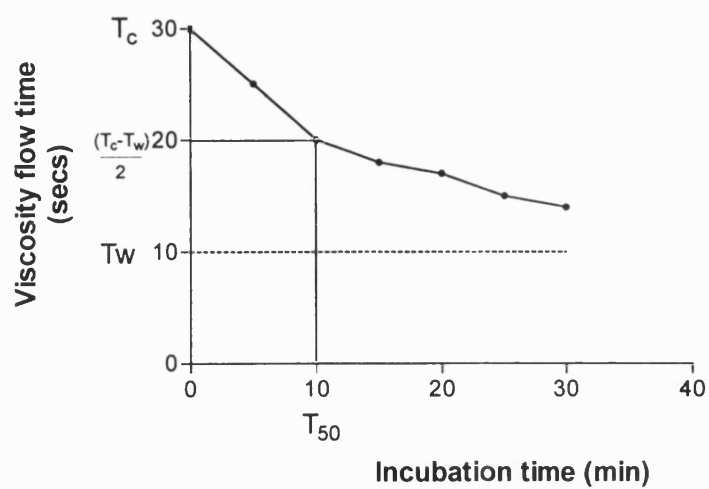


Fig. 5.9. Viscometric assay of Xyl1 xylanase activity. T_c = viscosity flow time for 4% birch wood xylan. T_w = viscosity flow time for water. T_{50} = time taken to reach a 50% decrease in relative viscosity.

Xyl1 was assayed viscometrically by incubation with 4% birchwood xylan. Viscosity measurements were taken every 5min for 30min and compared to a water control and substrate before digestion and the decrease in viscosity over time plotted (Fig. 5.9). This was repeated twice and from these data the time taken for a 50% reduction in relative viscosity was determined. A rapid reduction in viscosity was observed and a 50% reduction in viscosity (T_{50}) was reached after 10 minutes. This equates to 100 Relative Viscosity Units ($1000/t_{50}$) and confirmed that Xyl1 is endo-acting.

5.1.6 N-terminal sequencing of Xyl1

In order to obtain N-terminal protein sequence Xyl1 was blotted onto nitrocellulose and stained as described in materials and methods. The blot was sent to the Protein sequencing unit, Department of Biochemistry, University of Cambridge, for N-terminal sequencing and found to be N-terminally blocked. N-terminal blockage can be a property of the protein itself or it may occur during sample electrophoresis. To ensure that electrophoresis or blotting was not the cause of the blockage, certain steps were taken to eliminate this possibility, as recommended by Packman (1993). SDS-PAGE gels were cast the day before use to ensure complete polymerisation, as free acrylamide can block N-termini; 2mM thioglycollic acid was added to the upper well buffer, this migrates ahead of the protein and scavenges free radicals that may react with the N-terminus; 5mM DTT was included in the transfer buffer to prevent blockage during transfer. However, these steps may not be sufficient to eliminate N-terminal blocking during electrophoresis as one or more reactive species may be generated during the separation itself. A substance resembling acrylamide monomer is released during electrophoresis and this can react with primary amines, particularly at higher pH (Moos *et al.*, 1988). The Laemmli SDS-PAGE buffer system runs at an operating pH of around 9. Moos *et al.* (1988) used a neutral buffer system (MZE system 33.28.IV) at pH 7.28 which would be much less likely to allow the modifications to N-termini while still maintaining adequate ionisation of SDS-PAGE-protein complexes. Also, this buffer system does not contain glycine

which may interfere with sequence analysis. Using this method, combined with pre-electrophoresis with thioglycolic acid and transfer to PVDF membranes Moos *et al.* (1988) significantly reduced N-terminal blockage and allowed initial sequencing yields of 60-85% to be consistently obtained. The buffer conditions of the MZE 3328.IV system (Table 5.1) were used in conjunction with the other modifications but this failed to alter the N-terminal blockage. It was concluded therefore that the xylanase is naturally N-terminally blocked.

Constituent	1x Upper Buffer	1x Lower Buffer	4x Gel Buffer
TES (g)	10.07		
1 N Hcl (ml)		50.0	21.60
BisTris (g)	23.66	13.10	10.31
Final volume (ml)	1000	1000	100
pH (25°C)	7.25	5.9	6.61

Table 5.1 The composition of the pH 7.28 MZE 3328.IV buffer system (Moos *et al.*, 1988).

5.1.7 Generation of internal protein fragments

In order to circumvent the N-terminal blockage of Xyl1 attempts were made to digest the xylanase using a commercial protease to generate internal peptide fragments that could be sequenced. If enough internal sequence were obtained this might allow characterisation of the xylanase but would also provide useful protein sequence data for the design of oligonucleotide probes for future gene cloning attempts. Proteolytic degradation can be quite straightforward with samples of pure protein. However, the xylanase had not been purified and other proteins, particularly the trypsin-like protease were also present in fractions from the S-column. Any peptides generated might therefore be from contaminants if this sample was used, rather than Xyl1. Instead, the Xyl1 band was excised from an SDS-PAGE gel following electrophoresis and eluted from this gel slice. This pure protein could then be digested and subsequently electrophoresed to confirm digestion and a replicate gel blotted and fragments sequenced. The method

chosen was adapted from Packman (1993). Following electrophoresis the gel was stained and destained as usual. The 30kD band corresponding to the xylanase was then excised, diced into smaller pieces and then placed in a 1.5ml eppendorf. 1ml of 50mM Tris/Cl, pH8, 0.1% SDS was then added and the gel slice incubated in this buffer for 4h at 37°C. The buffer was then removed and replaced with fresh buffer, this was repeated to a total of 5 times and the 5ml of eluted protein pooled. This 5ml was then lyophilised and the residue resuspended in 100µl of dH₂O. The protein was then precipitated by the addition of 9 volumes of absolute ethanol at -20°C for 4h. The protein was then recovered by centrifugation for 10min at 12,000g and the pellet washed with 1ml of cold ethanol and then drained. The pellet was then resuspended in 15µl 50mM Tris pH8, 0.1%SDS and then 5µl of sequencing grade trypsin (Boehringer Mannheim Biochemica, modified to be more resistant to autolysis) at 1-5% w/w proteinase/protein in 1mmol/l HCl was added and incubated at 37°C for 3h. Following digestion, 5µl of SDS-PAGE loading buffer was added, the sample boiled and electrophoresed under the conditions outlined above and then blotted onto PVDF membrane and stained for sequencing. An example of this digestion is shown in Fig. 5.10.

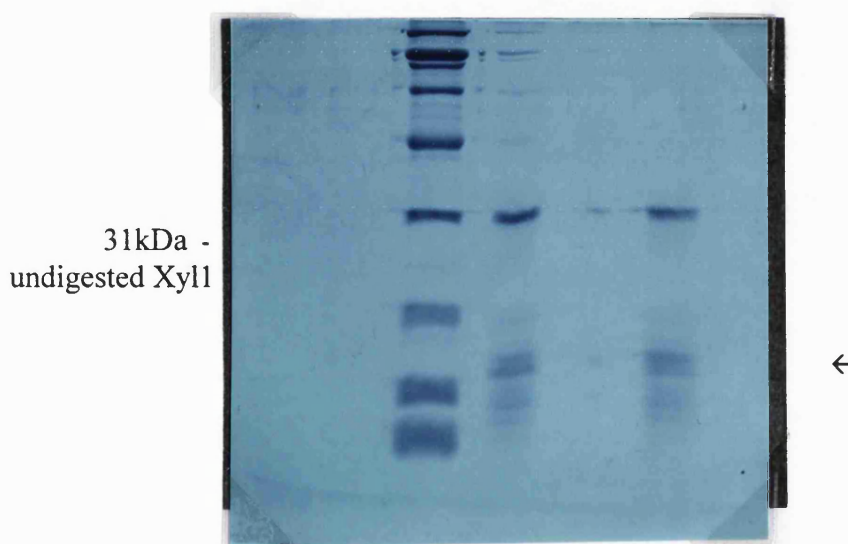


Fig.5.10. SDS-PAGE of Xyl1 following digestion with bovine trypsin at 37°C for 3h. A similar gel was blotted onto PVDF membrane and the two bands indicated by the arrow were used for sequencing attempts.

Fig.5.10 shows that digestion of Xyl1 was successfully achieved and a number of smaller protein bands were generated. However, only partial digestion took place as there was still a large amount of the original xylanase protein present (30kD). A similar gel was blotted and sent to the Cambridge protein sequencing unit. Only two bands (1,2 marked on Fig.5.10), once blotted had sufficient protein present for a sequencing attempt to be made. Band 1 did not sequence because of insufficient protein, which gave a low signal. Band 2 however was present in sufficient amounts, but was the N-terminally blocked fragment. Because of the increasing focus on the trypsin work and the apparent complexity of the xylanase system no further attempts to generate internal sequence were made.

5.1.8 Production of antibodies to Xyl1

Antibodies were raised to Xyl1 for use in detecting production of Xyl1 by *S.nodorum* during infection and also to distinguish this xylanase from others *in vitro*. Antibodies were raised to the xylanase from excised gel slices as described in materials and methods. To check the specificity of the antibody western blots of 100µg of crude culture filtrate were probed with the antibody at 1/10, 1/100, 1/500 and 1/1000. A single band of 30kD was detected with all of these dilutions (Fig.5.11) and no cross reaction with any other proteins was observed.

5.1.9 Carbon regulation of Xyl1

S.nodorum was grown on a range of carbon sources to investigate the specific regulation of Xyl1 using western blot analysis. Established biomasses were starved and transferred to the different carbons sources and cultures sampled at 24 and 48h and assayed with RBB-dyed xylan (Table 5.2).

Medium	Xylanase activity ($A_{595\text{nm}}$)	
	24h	48h
-C	0.18	0.15
1% xylose	0.45	0.22
1% oat spelt xylan	0.44	0.42
1% wheat cell wall	0.75	0.88
1% wheat cell wall + 1% glucose	0.16	0.34

Table 5.2. Xylanase activity produced by *S.nodorum* when grown on a range of carbon sources. Xylanase activity was assayed with RBB-dyed xylan.

As observed previously in section 5.1.1 xylanase production was greatest in cultures grown on wheat cell walls and was about twice that produced on oat spelt xylan. Only a very low amount of xylanase was produced in cultures grown on cell wall plus glucose at 24h but this increased at 48h, probably following glucose depletion. Again, xylanase production equal to that on oat spelt xylan was observed on xylose at 24h but this was considerably lower by 48h. In cultures grown with no carbon source only a very low level of xylanase activity was detected.

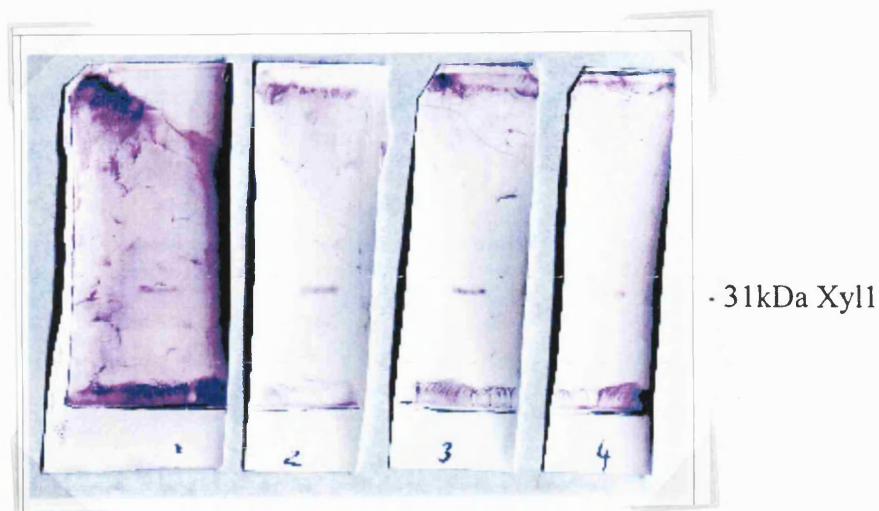


Fig.5.11. Detection of Xyl1 in crude 48h cell wall-grown culture filtrate with anti-Xyl1 antibody. 100 μ g of culture filtrate was electrophoresed using SDS-PAGE and blotted onto nitrocellulose. Blots were incubated for 1h with (1) 1/10, (2) 1/100, (3) 1/500 and (4) 1/1000 dilutions of the antibody. Antibody binding was detected with an alkaline phosphatase linked goat anti-rabbit antibody.

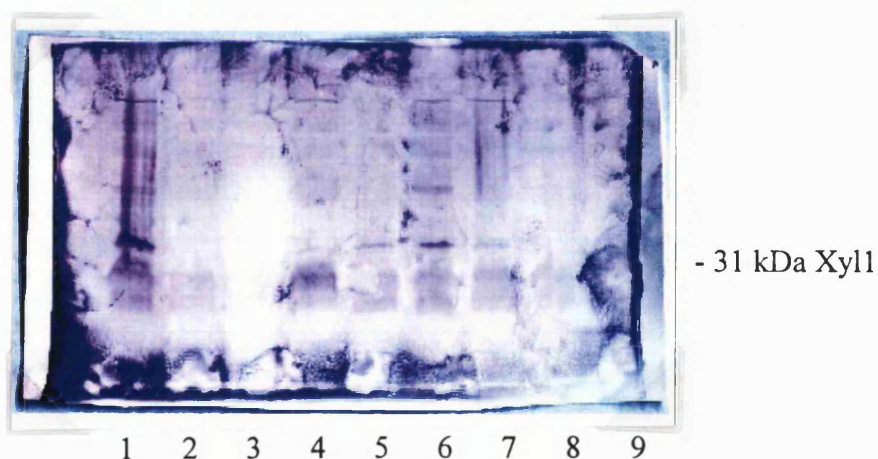


Fig.5.12. Regulation of Xyl1. *S.nodorum* was grown in a range of culture conditions and 100 μ g of culture filtrate was electrophoresed using SDS-PAGE and the presence of Xyl1 detected as described (Fig.5.11) using a 1/500 dilution of the anti-Xyl1 antibody. Cultures were (1) Wheat cell walls 24h; (2,3) Xylose 48h, 24h; (4,5) Oat spelt xylan 48h, 24h; (6,7) Wheat cell wall+1% glucose 48h, 24h; (8,9) -C-N 48h, 24h.

The antibody to Xyl1 was used to distinguish the production of Xyl1 in these cultures from total xylanase activity by western blotting (Fig.5.12). 100µg of each culture filtrate was electrophoresed in a 12% SDS-PAGE gel and blotted on to nitrocellulose. This membrane was then probed with a 1/500 dilution of anti-xylanase antibody and bound antibody detected with alkaline-phosphatase linked anti-rabbit antibody.

Xyl1 was not responsible for the low basal synthesis observed in cultures grown with no carbon source, neither was it present in cultures grown on xylose. It was present in low amounts at 24h on wheat cell walls plus glucose and increased at 48h. It was clearly present at 24h on oat spelt xylan and wheat cell walls but almost absent at 48h on oat spelt xylan. 48h wheat cell wall was not included because Xyl1 is known to be present at 48h because culture filtrate from this time point was used for its purification. It is clear from this work that Xyl1 is not expressed constitutively and is induced by polymers containing xylan, i.e. cell walls and oat spelt xylan. It was partly repressed by glucose and completely by xylose. Xylanase activity detected in derepressed cultures or in xylose cultures must be attributed to other xylanase isoforms. In this study, the antibody appeared to cross-react with some other bands, particularly a high molecular weight band under some conditions; this may be another xylanase or a particularly abundant protein. This cross reaction was not observed previously in section 5.1.8., but may have resulted here from allowing the alkaline phosphatase reaction to continue to develop for longer in this study for the detection of any Xyl1 protein in these different cultures.

5.1.10 Detection of Xyl1 in infected plant tissue

The aim was to determine whether Xyl1 was produced by *S.nodorum* during infection of wheat and whether it was present in the cirrus mucilage surrounding spores produced *in vitro*. A new more rapid method of generating infected plant material was developed for these studies, as described in materials and methods.

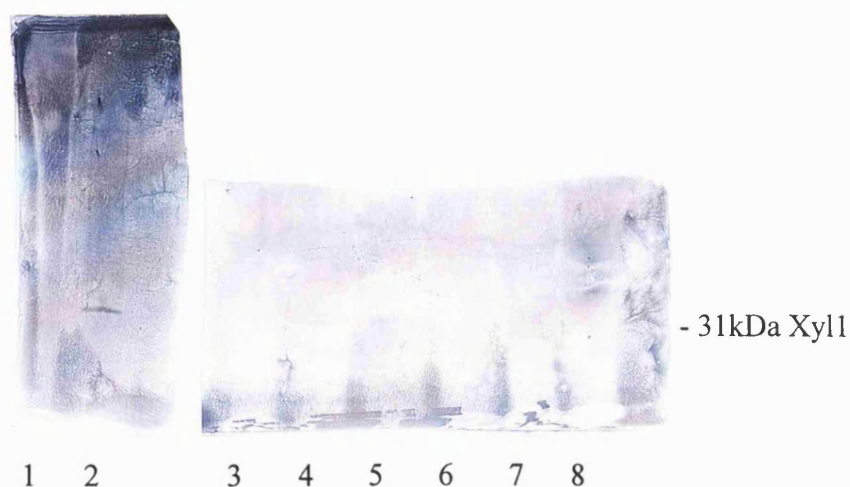


Fig.5.13. Detection of Xyl1 in infected plant material. 1mg of protein extract from infected and control plants was electrophoresed using SDS-PAGE and Xyl1 detected as described (Fig.5.11) following an overnight incubation with a 1/10 dilution of the anti-Xyl1 antibody. (1) 100 μ g cirrus mucilage; (2) 100 μ g of 48h cell wall grown culture filtrate; (3) day 7 control plants; (4) day 7 infected plants; (5) day 4 infected; (6) day 2 infected; (7) 24h infected; (8) day 2 control.

Leaves were inoculated by dipping into a suspension of 1×10^5 spores/ml and material was harvested at 24h, 2 days, 4 days and 7 days after infection. No symptoms were observed at 24h, at 2 days chlorotic spots appeared, by 4 days necrotic spreading lesions were present and by day 7 the leaves were moribund. Control plant material was harvested at day 2 and day 7. 1mg of protein from infected and control plant extracts were blotted onto nitro-cellulose following SDS-PAGE electrophoresis along-side 100 μ g of 48h 1% wheat cell wall culture filtrate as a control and 100 μ g of cirrus mucilage. Western blots were then probed with a 1/10 dilution of the xylanase antibody (Fig.5.13). The top half of the western blot from plant material was probed separately from the lower half because the high antibody concentration reacted non-specifically with abundant high molecular weight proteins.

A single protein band of approximately 40kDa was detected in both control and infected tissue. This may either be a plant xylanase or a particularly abundant

protein. No protein band corresponding to Xyl1 (30kDa) was detected in control tissue or in infected tissue at 24h or 2d after infection. However, a 30kDa band corresponding to Xyl1 was detected at 4 and 7 days after infection, coincident with the appearance of necrotic lesions and subsequent death of the plant. Xyl1 was not found in cirrus mucilage, so this high pI xylanase cannot be responsible for the activity previously detected in Chapter 3.

5.1.11 Effect of Xyl1 on wheat cell walls

5.1.11.1 Detection of reducing sugar release

The presence of Xyl1 *in planta* suggested that Xyl1 may play a role in host wall degradation during colonisation of wheat by *S.nodorum*. To determine whether Xyl1 was capable of degrading the wheat cell wall in the absence of other CWDE 5mg of wheat cell wall was washed with agitation for 1h with 50mM Tris-HCl pH 8.0. Following centrifugation at 12,000 g the buffer was removed and replaced with 950µl of fresh buffer and 50µl of Xyl1 (25µg of protein; 96nkats activity). Also included in the buffer was aprotinin at a final concentration of 10µM to inhibit SNP1. Controls contained 50µl of boiled protein sample. Cell wall was then incubated, with agitation and samples taken at intervals for 5h. Two samples were taken at each time point, centrifuged at 11,000 xg for 15min to remove undigested cell walls and the reducing sugar content of the supernatant measured using the Nelson-Somogyi reducing assay. Reducing sugar release was detected after 30min, and this increased linearly over time. Absorbance was converted to xylose equivalents and the percentage of the wall degraded calculated (Fig.5.14). 0.23% (w/w) of the wall was degraded after 30min, and this continued to increase until by 5h 1.3% of the wall had been degraded.

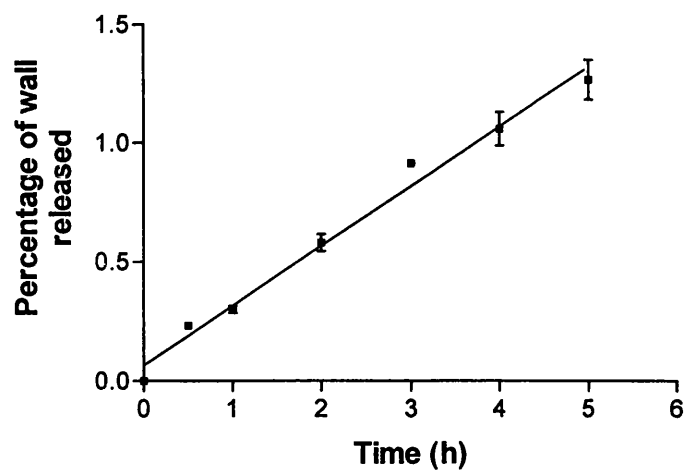
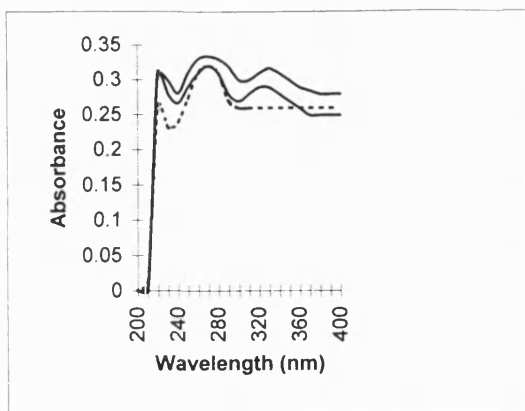


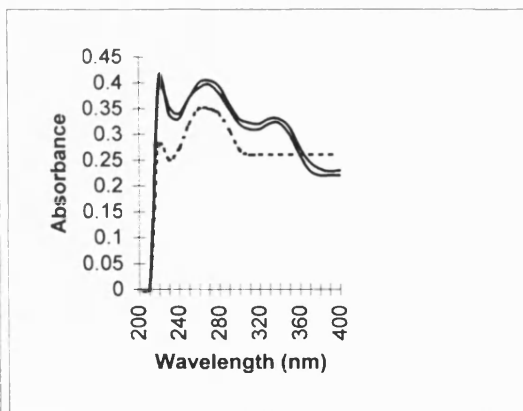
Fig. 5.14. Effect of Xyl1 action on wheat cell walls. Wall degradation was measured by determining release of reducing sugars (calculated as xylose equivalents). Error bars represent the standard deviation about the mean of three replicates.

5.1.11.2 Detection of phenolic-linked polysaccharides

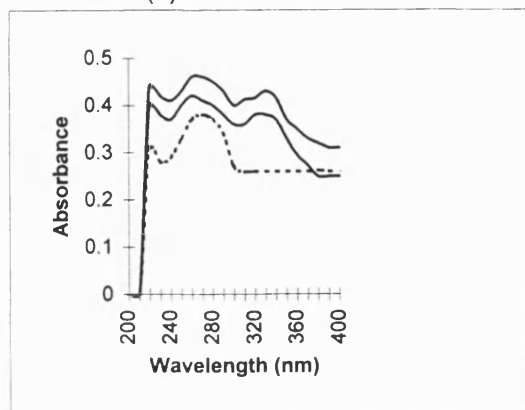
Phenolics absorb U.V. light in the 250-380nm range. The absorption maximum and shape of the absorption spectrum depends on the chemistry of the phenol and the pH of the solvent. Simple phenolics, e.g. tyrosine absorb maximally at about 250-280nm at pH 7 or below, and at 275-300nm in alkali owing to ionisation of the phenolic -OH group. Phenolics with a conjugated side chain, e.g. the cinnamic acid derivatives, absorb at longer wavelengths, typically 308-340nm, shifting to 330nm-380nm at higher pH (Fry, 1988). Arabinoxylan present in the wheat cell wall is known to possess ferulic acid and coumaric acid groups ester linked to the arabinose side chains. These groups are thought to be involved in cross-linking arabinoxylans in the cell wall (Brett and Waldron, 1990). The aim of this work was to see if any phenolic-linked oligosaccharides were released following Xyl1 action on the wall. Each of the samples generated above were scanned in the range 200-400nm before assaying for reducing sugars. The 200-400nm scans of each sample are shown in Fig.5.15. along side the control spectrum for ferulic acid ester linked to polysaccharide. An increase in absorbance in test samples was observed across the whole 200-400nm spectrum, compared to controls. The absorption spectrum over the 200-300nm range mirrored that of controls, consisting of two main peaks which increased in absorbance over time with Xyl1 treatment. However, due to the action of Xyl1, a new peak appeared which was not present in controls, in the range 300-350nm (max at 325nm). After 30min this peak was 0.05 A greater than in controls and after 5h this was approximately 0.2 A greater. The pure feruloyl ester at pH3, shown in fig. 5.15 has a very similar spectrum to that of a feruloyl-polysaccharide, and absorbs maximally around 325nm. This suggests that the increase in absorbance at 325nm observed may derive from ferulic acid-linked xylooligosaccharide fragments released by Xyl1 action. Samples also fluoresced blue under 366nm U.V. light which is another characteristic of ferulic acid; *p*-coumaric acid is invisible at this wavelength (Fry, 1988).



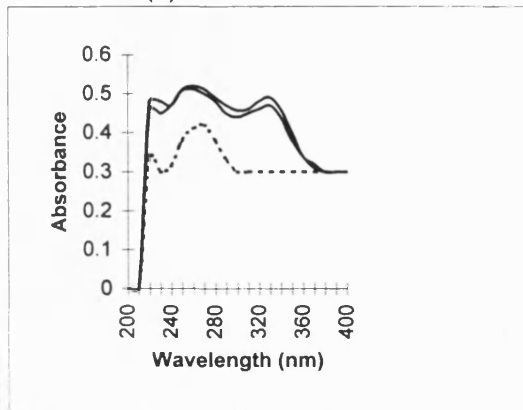
(a)



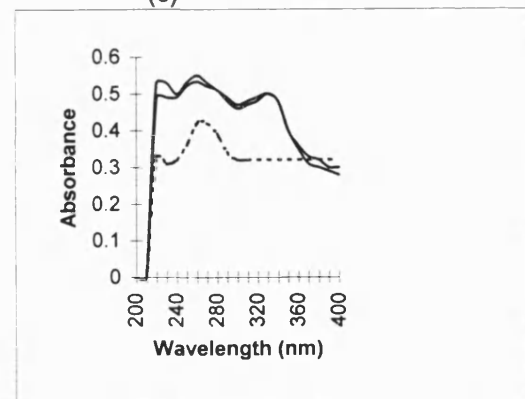
(b)



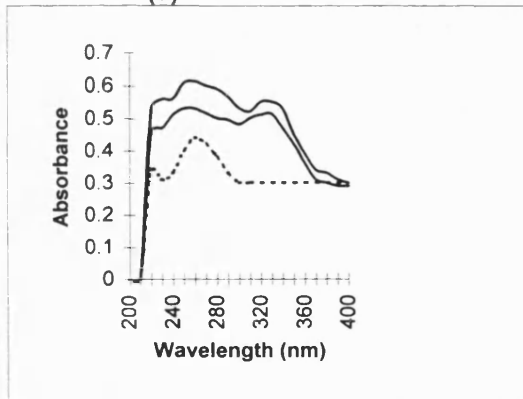
(c)



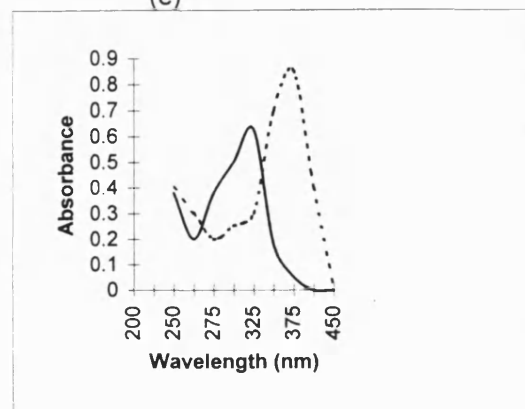
(d)



(e)



(f)



(g)

Fig.5.15. The effect of Xyl1 on wheat cell walls. 5mg of wheat cell walls was incubated with(-) or without (---) 50 μ l of Xyl1 for (a) 30min, (b) 1h, (c) 2h (d) 3h, (e) 4h, (f) 5h. (g) Control spectrum of ferulic-acid linked polysaccharide at pH3 (-), and at pH 11 (---) reproduced from Fry (1988).

5.1.12. Effect of Xyl1, with and without SNP1 on tritordeum suspension cells

A number of cell wall degrading enzymes are known to be toxic to plants directly or *via* oligosaccharides released by their action (Walton, 1994). The purpose of this investigation was to determine whether Xyl1, which was known to be able to degrade the plant cell wall, would be capable of killing tritordeum suspension cells, alone or in combination with the trypsin-like protease. Pure SNP1 alone, at very high concentrations had already been shown to be incapable of killing the cells, despite its ability to degrade the wall (Chapter 4). The co-purification of Xyl1 and SNP1 suggests that these two enzymes have very similar properties, both have high pIs, alkaline pH optima and similar molecular weights (31 and 25 kDa respectively). These similar properties suggested that they may be secreted together *in vivo* and given their abilities to degrade the plant cell wall could act synergistically in achieving wall degradation. However, SNP1 was detected throughout infection by western blotting while Xyl1 was only present during later stages of infection suggesting that synergistic action/ co-secretion of these two enzymes was not occurring.

To test whether any of the range of cell wall degrading enzymes produced by *S.nodorum* in combination with cell wall fragments could kill suspension cells, crude culture filtrate (dialysed) was also tested. Because of limited availability of partially purified Xyl1 the concentrations used were 25µg protein (96nkats Xyl1; 110nkats SNP1), 50µg (192nkats Xyl1; 220nkats SNP1) and 500µg (1.92mkats Xyl1; 2.2mkats SNP1) with and without aprotinin at 10µM final concentration (known to completely inhibit SNP1 activity). Controls were boiled protein, control assay medium and aprotinin only. 48h cell wall culture filtrate was used at 2.5ml (750µg protein) and 5ml (1.5mg protein). Each of the samples were freeze-dried and then resuspended in 1ml of assay medium (0.5mM MES, 0.5mM CaCl₂, 0.5mM K₂SO₄ and 175mM mannitol and adjusted to pH 5.7). Samples in triplicate were added to 0.05g of suspension cells and incubated at 25°C, 70rpm for 48h. 100µl samples of cells were taken from each of the three replicates for each treatment and tested for viability using FDA at 2h, 12h, 24h, and 48h. None of the treatments caused any significant death of the suspension cells under these conditions.

5.1.13 The effect of anti-Xyl1 antibody on disease symptoms

The aim of this experiment was to determine if inhibition of Xyl1 activity with the antibodies raised against Xyl1 could prevent disease caused by *S.nodorum*. This was tested by infiltrating the leaf with xylanase antibody and also by mixing it with spores applied on the leaf surface. Neat antibody was first tested against the partially purified xylanase to test if it inhibited xylanase activity. An 80% reduction in activity was observed *in vitro* against 2.5µg of protein, corresponding to 0.66 A at 595nm after 1h; however at 5µg only a 60% reduction was observed. Spores were mixed with neat antibody (1:1) at a final concentration of 1x10⁶ spores/ml and applied to the leaf surface in 5µl droplets. Leaf bridges were made on benzimadozole agar as described in materials and methods and the leaves were incubated at 20°C under 12h dark/light cycle to allow symptoms to develop. Symptoms were assessed using the disease score described in materials and methods at 4,6, and 8 days after inoculation. No significant reduction in disease was observed between control and infected plants from 10 replicates (Table 5.3) and no effect of anti-Xyl1 on spore germination was observed.

Time	Treatment	
	Disease Score (SD)	
	Xyl1-antibody	dH ₂ O
4d	0.4 (0.8)	1 (1.3)
6d	2.8 (1.17)	1.75 (0.96)
8d	3.2 (1.3)	3.0 (0.7)

Table 5.3. The effect on disease score of mixing Xyl1 antibody with spores. Antibody was mixed with 1x10⁶ spores/ml and 5µl droplets applied to leaves. Disease was assessed at days 4, 6 and 8. Disease score was 0 - no disease
1- water soaking, 2 - chlorosis, 3 - necrotic spots in a chlorotic region, 4 - necrotic lesion, 5 - spreading necrotic lesion.

Leaves were also infiltrated with antibody to see if this could suppress infection. Antibody was infiltrated at a 1/10 dilution as described in materials and methods. Inoculation, incubation and assessment were as before. Again, no significant reduction in disease was apparent (Table 5.4).

Time	Treatment	
	Disease Score (SD)	
	Xyl1 antibody	dH ₂ O
4d	2.1 (0.51)	1.5 (0.34)
6d	3.6 (0.48)	4.2 (0.4)
8d	3.8 (0.8)	5 (0)

Table 5.4. The effect on disease score of infiltration of leaves with Xyl1-antibody prior to inoculation. Scores are the mean of ten replicates.

5.2 Discussion

5.2.1 Xylanase regulation

Regulation of total xylanase production was studied by assaying liquid cultures grown on a range of carbon sources against RBB-dyed xylan. This substrate is specific for endo-xylanases but will obviously not distinguish between different xylanase isoforms which may be differentially regulated. Production of xylanase was induced by substrates containing xylan *ie* wheat cell walls and oat spelt xylan and production was greatest on wheat cell walls. A basal synthesis was observed on the non-inducing polymer, pectin and this activity as well as that on wheat cell walls was repressed by glucose. This observed repression of xylanase activity by glucose in the presence and absence of wheat cell walls is consistent with the work of Lehtinen (1993) who also observed glucose repression of *S.nodorum* xylanase activity. Xylanase activity therefore showed a typical regulation for a CWDE of substrate induction and carbon repression (Cooper, 1983). Interestingly, total xylanase production was not catabolite repressed by xylose but was instead induced by it.

The availability of the antibody to Xyl1 of *S.nodorum* enabled the specific regulation of this one isoform to be studied. No Xyl1 protein was detected in cultures containing no carbon source, although xylanase activity was detected. This activity may be the derepressed or basal synthesis observed previously in pectin cultures but it cannot be attributed to Xyl1. Xyl1 was present in cultures grown on glucose at low levels at 24h and higher levels at 48h, presumably due to glucose depletion. Earlier time points would give a clearer picture of glucose regulation. Only very low levels of Xyl1 were produced on oat spelt xylan in contrast to high levels observed on wheat cell walls. This may explain the difference in overall xylanase production detected on the two substrates, especially if other isoforms follow a similar pattern. Xyl1 was completely repressed by 1% xylose and again this may account for the lower level of total xylanase activity in these cultures compared to wheat cell walls. This work

highlights the fact that the xylanase activity detected in cultures is probably composed of a number of isoforms that may be differentially regulated. Xyl1 therefore requires a substrate for induction, was induced by polymers containing xylan and was catabolite repressed by xylose and possibly glucose.

Xylan cannot act as the direct inducer of xylanases because its large molecular size prevents uptake by the fungus. Instead, products of xylan catabolism such as xylobiose, xylose and metabolites of their further catabolism such as xylitol and xylose could function as inducers instead. Low basal synthesis of xylanase and xylosidase therefore may release these inducing xylooligosaccharides and xylose which may enter the cells and mediate induction of more xylanolytic enzymes. Xylose appeared to be the inducer of at least some xylanase isoforms but not Xyl1. This may account for the lower level of xylanase production on xylose compared to cell wall cultures. Xylose may still be the inducer of Xyl1 but perhaps at a lower concentration than was used in this work. For example, xylose induction and glucose repression was observed for the xylanases of *Aspergillus sydowii* (Ghosh and Nanda, 1994). However, these cultures contained only low levels of xylose (0.1% compared to 1% in this work) which may have prevented any repressive effect. Similarly, low levels of xylose were found to be inducing when released slowly into the cultures of *Verticillium albo-atrum*, mimicking their slow release *in vivo*, while in unrestricted cultures (1% xylose) production was repressed (Cooper and Wood, 1975). Alternatively, the lower level of total xylanase production and absence of Xyl1 on xylose may be due to true catabolite repression by xylose. Xyl1 may instead be induced by xylooligosaccharides, such as xylobiose, as reported for the yeast *Cryptococcus albidus* (Biely, 1985).

Independent regulation of xylanase isoforms has been observed in a number of systems but obviously requires either the use of a specific antibody to the xylanase of interest or more commonly studied are changes in transcript at the RNA level in order to distinguish individual isoforms within a complex mixture. In *M.grisea* the mRNA of one of two xylanases studied is produced at tenfold

higher levels than the other when grown on rice cell walls, suggesting that they are regulated at the level of gene expression (Wu *et al.*, 1995). The relative concentration of each transcript also varied over time suggesting that they are independently regulated. In a more detailed study on the nature of xylanase inducers the two genes encoding two different xylanases of *Trichoderma reesei*, *xyn1* and *xyn2* were found to be differentially regulated. *Xyn1* was induced by xylose while *xyn2* was induced by xylobiose (Zeilinger *et al.*, 1996). However, these authors suggest that the difference in regulation may still be mediated by xylose at the level of intracellular concentration. The concentration will be high when the fungus is grown on xylose compared to xylobiose because uptake of xylose will be quicker than following hydrolysis of xylobiose. Hence these different isoforms may have different thresholds of catabolite repression by xylose. The importance of β -xylosidase in this process therefore warrants further investigation.

Differential regulation by xylose may also explain the lower production of total xylanase and Xyl1 when *S.nodorum* was grown on soluble oat spelt xylan compared to insoluble wheat cell walls. Slower degradation of insoluble wheat cell walls may have prevented an accumulation of repressive levels of xylose. Alternatively, the difference in total xylanase production may reflect differences in xylan structure between the two substrates. Wheat arabinoxylan is known to be more highly substituted with arabinose than oat spelt xylan (Izydorczk and Biliaderis, 1995) and hence may require more xylanases with different substrate specificities to achieve its degradation. For example, the presence of arabinose substitutions may induce xylanases capable of releasing arabinose from the xylan backbone. One such specific arabinoxylanase has been isolated from *Trichoderma koningii* (Wood and McCrae, 1986a).

Different xylanase isoforms have previously been shown to be induced by wheat cell walls and oat spelt xylan in cultures of *S.nodorum* (Lehtinen, 1993). Of thirteen isoforms detected in total, eight were present on both wheat cell walls and oat spelt xylan, four were only present on wheat cell walls and one was only

present on oat spelt xylan. The ability of different xylanases to degrade differentially insoluble and soluble xylan substrates and produce different hydrolysis products as a result has been observed for two xylanases of *Streptomyces* sp. A451 (He *et al.*, 1993). XylII was more effective at degrading the soluble fraction of oat spelt xylan than the insoluble fraction, while XylIII showed the reverse property. These xylanases also generated different degradation products; XylII produced xylotriose while XylIII produced xylotetraose.

All xylanase production by *S.nodorum* on wheat cell walls was repressed by glucose. Complete repression was observed until 24h after which low xylanase activity was detected. This later induction of xylanase activity was most probably due to glucose depletion relieving glucose repression. Most xylanases are glucose repressed, including those from *M.grisea* and *C.carbonum* (Wu *et al.*, 1995; Apel, *et al.*, 1993). Basal synthesis of xylanase 1 of *T.reesei* is repressed in the presence of glucose alone, however its induction by xylan and xylose is not, such that the presence of either xylan or xylose can overcome any glucose repression (Mach *et al.*, 1996).

There is only limited knowledge of the molecular basis of glucose repression in fungi. Repression is mediated by direct negative control of target promoters by a DNA-binding repressor protein such as Mig 1 from yeast or the related CREA repressor in *Aspergillus* (Ronne, 1995). The involvement of a CREA in the carbon catabolite control of xylanase has been shown for *A.nidulans* and *A.tubigensis* (DeGraaff *et al.*, 1994). In *A.tubigensis* carbon-catabolite repression of the xylanase encoding gene *xlnA* is thought to be controlled at two levels, directly by repression of xylanase gene expression by CREA and indirectly by repression of the expression of a transcriptional activator. This mechanism is similar to the double lock mechanism for regulation of the *alcA* gene expression in *A.nidulans*. Here CreA competes with the positive regulator AlcR for DNA-binding sites, and the presence of CreA prevents AlcR binding (Matthieu and Felenbok, 1994). A similar repressor protein, Cre1 has been identified in

Trichoderma reesei and this has been shown to bind to the promoter of the xylanase 1 (*xyn1*) gene (Mach *et al.*, 1996). In contrast to *A.tubigensis*, Cre1 binding to the promoter causes repression by direct interaction with the RNA-polymerase II initiation complex which prevents transcription. However, as mentioned above, this glucose repression can be overcome in the presence of xylan or xylose. Induction by xylan or xylose somehow prevents the interaction between Cre1 and the RNA-polymerase II initiation complex from occurring. Induction is also thought to act by the binding of a xylan-specific transactivator to the promoter and the initiation complex (Mach *et al.*, 1996). It is not known whether this requires a functional inactivation of the Cre1 complex or a gain in binding strength of the transactivator complex (Zeilinger *et al.*, 1996).

5.2.2 Xylanase multiplicity

The regulation studies found that Xyl1 was not responsible for the xylanase activity detected on xylose or in derepressed cultures, indicating that other xylanase isoforms must be produced by *S.nodorum*. Isoelectric focusing studies showed that a number of different isoforms of xylanase are produced by *S.nodorum* when it is grown on wheat cell walls. The exact number was not determined but the Rotofor™ separation showed them to have pIs in the range 3.5-7.0. Further optimisation of overlay gels is required in order to determine the exact number of isoforms. This may be assisted by using narrower range isoelectric focusing gels in order to achieve better separation of large numbers of isoforms with very similar pIs prior to detection with overlay gels. Both the Rotofor and overlay methods failed to detect the high pI xylanase activity (Xyl1), later discovered by chance. The reason for this is unclear. It may be that the high pI xylanase is unstable at low pH, for isoelectric focusing samples were dialysed against distilled water at pH 4-5.0 (because dialysis against buffer interferes with the gradient). This pH may have been too low, particularly because the pH optimum of Xyl1 was 7.0, and hence some activity may have been lost after lengthy dialysis at a lower pH. Also, it is not possible to know how much of the total xylanase activity can be accounted for by Xyl1; it may be only a minor

isoform and the conditions mentioned above may have made it undetectable. The range of isoforms detected in this study using isoelectric focusing are consistent with the results of Lehtinen (1993) who found 13 different isoforms of xylanase from wheat cell wall grown culture filtrates of *S.nodorum*. These isoforms had pIs of: 3.6, 3.8, 3.9, 4.0, 4.2, 4.7, 4.8, 4.9, 5.5, 5.8, 6.0, 6.1, 6.2 and no high pI forms were detected. The presence of so many isoforms with similar pIs possibly explains the difficulties found in obtaining a clear overlay gel. Lehtinen (1993) used more sensitive IEF gels with the pH ranges 2.5-5 and 5-8 to separate further xylanase isoforms detected in gels with the pH range 3.5-9.5. The presence of numerous isoforms contrasts with the work of Magro (1984) who only found a single xylanase isoform of pI 5.2 when *S.nodorum* was grown on wheat cell walls. Column isoelectrofocusing by Magro (1984) was performed over 48h which may have resulted in some loss of isoforms. However, such a large discrepancy suggests that at least some of these isoforms may be artefactual, and could be due to proteolysis of the xylanases in the culture medium or as has been observed for cellulases may result from ampholyte binding (Goyal *et al.*, 1991). However, in the study by Lehtinen (1993) only low levels of protease were detected and profiles remained the same with different cultures and different ampholytes. It is likely then that *S.nodorum* produces a large number of xylanase isoforms that went undetected by Magro (1984). An established biomass transfer system was used in this work to try to reduce the number of isoforms that may have arisen due to proteolysis. In the study by Lehtinen (1993) *S.nodorum* was grown in liquid culture over 14 days compared to 48h in this work. It would be expected that proteolysis is much more likely during such a long culturing period. However, the results found here (based on a short culture period) indicate that a number of isoforms are produced by *S.nodorum*.

Xylanase multiplicity is extremely common and has been reported for a number of micro-organisms (Wong, 1988). The culture filtrate of *Aspergillus niger* contains 15 different isoforms, while that of *Trichoderma viride* contains 13 (Biely *et al.*, 1985b). The plant pathogenic fungi *C.carbonum* and *M.grisea* are capable of producing at least four and five forms of xylanases respectively (Apel-

Birkhold and Walton, 1996; Wu *et al.*, 1997). These apparent isoforms may arise through a number of mechanisms. Firstly, they may not be true xylanases and xylanase activity may be a secondary enzyme function. A number of cellulases have been shown to have xylanase activity, for example both xylan and cellulose compete for the active site of a cellulase from *T.viride* (Wong, 1988). Xylanase heterogeneity may arise from post-translational modifications, such as differential glycosylation or proteolysis or both. Hence de-glycosylation of the xylanases of *S.nodorum* before isoelectric focusing may have reduced the number of isoforms detected. Most xylanases are glycosylated and some are translated as precursors with peptide signal sequences (Wong, 1988). Some xylanase forms therefore may be precursors of, or degradative products from other xylanases. For example, *Gaeumannomyces graminis* produces two xylanases of different sizes but with identical N-terminal sequences when grown on xylan (Southerton *et al.*, 1993). Their identical N-terminal sequences suggests they are products of the same gene and arise through post translational modification. Neither xylanase was glycosylated so it is likely that the 26.5 kDa protein is a product of proteolysis of the 28kDa protein. Different isoforms may also arise if a xylanase is complexed to other enzymes such as cellulase or other xylanases. These complexes may have a different pI from their individual component enzymes. For example, a pI 5.7 complex isolated from *T.reesei* contains a xylanase with a pI of 6.0 when separated from the cellulase and β -glucosidase components (Wong, 1988). Multienzyme complexes of cellulases are known as cellulosomes and more recently an analogous structure, a xylanosome, has been reported in the rumen anaerobe *Butyrivibrio fibrisolvens*. This complex has a molecular weight greater than 669kDa and is composed of 11 protein bands showing xylanase activity (Lin and Thomson 1991). Finally, apparently different isoforms may arise through changes in pI during storage, for example, xylanase B from *T.harzianum*, an enzyme that changes pI from 8.5 to 7.2 upon storage in ethanolamine buffer at pH 9.4 (Wong, 1988). In these cases therefore xylanase multiplicity may be due to protein aggregation or protein instability.

However, as well as artefactual multiplicity, many xylanase isoforms are distinct gene products and multiple xylanase genes have been detected in many micro-organisms. For example, *M.grisea* has at least two xylanase encoding genes and *C.carbonum* at least three (Wu *et al.*, 1997; Apel-Birkhold and Walton, 1996). The production of different isoforms by different genes suggests that these individual xylanases have different properties that contribute to overall xylan degradation. These xylanases have indeed been found to have different properties and these appear to be conserved between different micro-organisms, perhaps suggesting a common xylanolytic system. Amino acid sequence analysis, coupled with hydrophobic cluster analysis has enabled the evolutionary relationships of xylanases from different micro-organisms to be established (Henrissat and Bairoch, 1996). On this basis two main groups exist, groups 10 and 11, conserved on the basis of molecular weight, substrate specificity and isoelectric point. Group 10 are high molecular weight and generally low pI while group 11 xylanases are low molecular weight with either acidic or alkaline pI (Torronen and Rouvinen, 1997). Xylanases in group 10 have a lower substrate specificity than group 11 and family 10 xylanases are better at cleaving glycosidic linkages in the xylan back bone closer to substituents. They can also hydrolyse the oligosaccharides released by family 11 xylanases (Biely *et al.*, 1997).

The catalytic differences between the two families suggests a synergism exists between these endo-xylanases in achieving xylan degradation. It is already known that many enzymes contribute to xylan degradation such as α -glucuronidase, acetylcetase, arabinosidase and phenolic acid esterases in removing side groups and relieving steric hindrance (Wong, 1988). Endoxylanase multiplicity suggests that these enzymes have evolved to cleave specific linkages in the xylan molecule that may be inaccessible to other enzymes. Co-operation between xylanases in achieving xylan breakdown has been observed for some systems including *T.harzianum* (Wong, 1988). The three xylanases produced by this fungus did not cooperate in the hydrolysis of arabinoxylan but co-operation between all three was required to achieve maximal

hydrolysis of deacetylated and acetylated xylan. Enzyme inactivation or product inhibition were not limiting factors in hydrolysis which suggests that the glucuronosyl and acetyl substituents required xylanase multiplicity. The identification that the NodB domain in xylanase D from *Cellulomonas fimi* functions as a deacetylase and releases acetyl groups from acetylated xylan suggests that this domain may work synergistically with the endo-xylanase domain in achieving xylan degradation (Laurie *et al.*, 1997). It has also been suggested that some xylanases may also exhibit significant arabinosidase activity. One endoxylanase from *Fibrobacter succinogenes* releases arabinose from oat spelt xylan and rye flour arabinoxylan, directly from the xylan backbone, before hydrolysis of the xylan to xylooligosaccharides (Matte and Forsberg, 1992).

Multiple xylanases with different physico-chemical properties may also allow micro-organisms to use a wider range of substrates, for example during saprophytic growth or on alternative hosts by plant pathogens. They may therefore contribute to determining host and/or tissue specificity in these pathogens and xylanolytic systems with lower multiplicity may be inferior to those with higher multiplicity. Alternatively, multiple xylanases may play a role in hydrolysis of other xylose containing polysaccharides or of secondary substrates. The related debranching and/or transferase activities of some xylanases may be their primary function and may be important in disrupting fibre integrity and exposing other lignocellulosic components to other hydrolases. Finally, some of these may be functionally redundant isoforms that have arisen during evolution.

The conclusion of this work was that the xylanases of *S.nodorum* appear to be complex, consisting of a number of different isoforms. Resolving the system genetically will be a difficult task but the partial purification of one of these xylanases provides a starting point.

5.2.3 Characteristics of a partially purified high pI xylanase (Xyl1)

A high pI xylanase (Xyl1) co-purified with the trypsin-like protease, SNP1 during preliminary cation exchange chromatography. It is interesting that Xyl1 was stable in the presence of the *S.nodorum* trypsin-like protease, when it was susceptible to attack by bovine trypsin, the commercial trypsin used in trying to generate internal protein fragments. These two proteases may have sufficiently different properties, such as substrate specificity that enables one but not the other to attack Xyl1. Alternatively, it may be a particular property of Xyl1 that makes it resistant to attack by the *S.nodorum* protease. In general, glycosyl hydrolases are unusually resistant to proteolytic attack and thermal inactivation (Fontes *et al.*, 1995). Calcium ions are known to play an important role in conferring structural stability on proteins and have been found to protect the xylanase of *Pseudomonas fluorescens* subsp. *cellulosa* from proteinase inactivation and thermal unfolding (Spurway *et al.*, 1997). Calcium ions bind into a calcium binding domain but do not alter the activity of the enzyme hence their presence is solely for stabilisation of the protein. Removal of the calcium binding site from this enzyme made it susceptible to degradation by chymotrypsin. Xyl1 may have such a calcium binding site and this may have been the cause of difficulties in digesting the xylanase for generation of internal fragments.

Attempts to generate N-terminal sequence from Xyl1 suggested that it was naturally N-terminally blocked. N-terminal blockage arises due to modification of the N-terminal group with formyl, acetyl or other acyl groupings or conversion to a pyroglutamyl residue. It occurs frequently and up to 50% of proteins may be blocked in this way (Aitken *et al.*, 1989). Such polypeptides are refractory to the Edman degradation chemistry so they need to be removed by mild acid hydrolysis or enzymic hydrolysis eg. with pyroglutamate aminopeptidase or acyl-amino acid-releasing enzyme, before sequence can be obtained (LeGendre *et al.*, 1993). These treatments work best with a protein in solution, and better still with peptides, as whole proteins are more resistant to treatment (Packman, 1993).

Alternatively, proteins can be enzymatically digested using commercial proteases into smaller peptides and internal sequence obtained; once again this is more successful with proteins in solution. Unfortunately Xyl1 had not been purified and so attempts were made to digest Xyl1 following elution from SDS-PAGE gel slices. There are a number of problems with this type of approach. The ratio of the commercial protease to the protein of interest is very important to ensure optimal activity of the protease. It is difficult to quantify the amount of xylanase in the starting impure fraction and then estimate how much of the protein is eluted from the gel slice and recovered by ethanol precipitation. This obviously varies every time the procedure is performed and only a limited amount of partially purified Xyl1 was available for this work which restricted the number of optimisation studies and made removal of aliquots for protein estimation at each stage impossible. Another problem is that once digestion has been optimised more protein is lost during transfer to the PVDF membrane and this may lead to insufficient yields for sequencing. An alternative approach to electro-blotting the peptides would be to purify them using reverse phase-HPLC (Stone and Williams, 1993). Future work should focus on finding a superior purification method for the xylanase and generating a large amount of pure protein. This could then be digested with commercial proteases in solution and the resultant peptides either electro-blotted or purified on HPLC. Digestion with a range of commercial proteases with different substrate specificities, such as chymotrypsins should also be tried to see if their action generates a better range of peptides. Alternatively peptides can be generated using chemical hydrolysis such as CNBr digestion or acid hydrolysis (Packman, 1993). One of the xylanases produced by *Magnaporthe grisea*, XYN33, was N-terminally blocked (Wu *et al.*, 1995). Internal peptides of this xylanase were generated by CNBr treatment in 70% formic acid for 20h at room temperature. The digested proteins were then fractionated by high-pressure liquid chromatography on a reverse-phase column and the purified peptides sequenced. One of the xylanases of *C.carbonum* is also N-terminally blocked and internal sequence was generated by tryptic digests (Holden and Walton, 1992)

Xyl1 has a molecular weight of 30kDa and a pI of at least 10. It is active over a range of temperatures, and was still active at 80°C, but has an optimum of 50°C. It is active over the pH range 5.0-9.0 but is optimally active at pH 7.0. Xyl1 is an endo-acting xylanase, as demonstrated by viscometry. All these characteristics suggest that this may be a family 11 type xylanase, although this cannot be confirmed without protein sequence data. Xylanases in family 11 are generally from filamentous fungi, have a low molecular weight and can have high pIs. The alkaline xylanases from family 11 tend to be active in the pH range 4.0-8.0 compared to a narrower pH range of 3-6 for the acidic xylanases (Torrönen and Rovinen, 1997). The low molecular weight is similar to that of many *Trichoderma* species (Lappalainen, 1986; Tan *et al.*, 1987), and the plant pathogenic fungi *M.grisea* (Bucheli, *et al.*, 1990), *Bipolaris sorokiniana* (Peltonen, *et al.*, 1994) *C.carbonum* (Holden and Walton, 1992) and *Helminthosporium turcicum* (Degefu *et al.*, 1995). The high pI of greater than 10 is similar to xylanases from some other fungal plant pathogens e.g. *C.carbonum* (Holden and Walton, 1992), *B.sorokiniana* (Peltonen *et al.*, 1994) and *M.grisea* (Wu *et al.*, 1995). The temperature optimum of 50°C is typical for fungal xylanases (Dekker and Richards, 1976), including *G.graminis* (Southerton *et al.*, 1993) although some are higher eg. *Bipolaris sorokiniana* xylanase has a temperature optimum of 70°C (Peltonen *et al.*, 1994). The pH optimum of 7.0 is relatively unusual as most fungal xylanases are most active between 3.5 and 5.5 (Dekker and Richards, 1976). However, a few xylanases with higher pH optima have been found for example the xylanase from *H.turcicum* has a pH optimum of 5.5-6.5 (Degefu *et al.*, 1995).

5.2.4 Detection of Xyl1 in planta

Xyl1 was detected in infected plant material by western blot analysis at days 4 and 7 after infection. The appearance of this enzyme coincided with the appearance of necrotic symptoms, consistent with the timing of detection of xylanase activity in earlier work (Chapter 3). This clearly demonstrates the expression of this enzyme by *S.nodorum* during infection of wheat and it is

therefore conceivable that it plays a role in wall degradation during growth through the plant by the fungus during mid to late stages of infection. Xylanases are thought to be important for pathogens of cereals because of the abundance of arabinoxylan in the wheat cell wall (Cooper *et al.*, 1988). Xylanase activity was previously detected in wheat infected by *S.nodorum* six days after inoculation (Magro, 1984). This xylanase activity was characterised as a single activity with a pI of 5.2 corresponding to the single isoform detected *in vitro* in this same study. It is clear from the work presented here that other forms exist *in vitro* and at least one other, Xyl1, is present *in planta*. Xylanase activity has been detected in wheat infected by a number of other fungi, including *G.graminis* (Southerton *et al.*, 1993), *Pseudocercospora herpotrichoides* (Mbwaga *et al.*, 1997) and *R.cerealis* (Cooper *et al.*, 1988). Western blot analysis has been used to detect cell wall degrading enzymes of pathogen origin during infection of the host plant in a number of systems, including the detection of an endopolygalacturonase from *Cochliobolus sativus* during infection of barley (Clay *et al.*, 1997). In the same study the enzyme was localised to its target cell wall polysaccharide with immuno-gold labelling of the anti-PG antibody visualised by electron microscopy. Corresponding loss of polygalacturonic acid in the vicinity of the invading pathogen was also observed, suggesting the involvement of the endo-PG in host penetration by the fungus. The availability of an antibody to Xyl1 now makes a similar approach possible to assess the localisation of Xyl1 during infection of wheat by *S.nodorum*.

The western blot analysis failed to detect Xyl1 in the cirrus mucilage, hence Xyl1 is not responsible for the xylanase activity detected by conventional assay. This suggests that at least one other xylanase isoform, possibly one of those detected *in vitro* must be present in the cirrus mucilage and may play a role in early infection. This is consistent with one theory on xylanase multiplicity that different isoforms may have different roles to play at different stages in the infection process. The failure of the anti-Xyl1 antibody to prevent disease is also consistent with its production in mid to late stages of infection. Application of antibody with spores would be unlikely to have any effect because Xyl1 is not

expressed at this time. Similarly, the use of antibody infiltration was probably unsuccessful because by the time Xyl1 was produced *in planta* the antibody would probably have been degraded by the plant or *S.nodorum*. It may be possible to infiltrate leaves again, later in infection to see if this could prevent disease by inhibiting Xyl1. Failure to reduce disease with the anti-Xyl1 antibody may have been due to incomplete inhibition of Xyl1. *In vitro*, neat anti-Xyl1 antibody failed to inhibit completely xylanase activity, hence if Xyl1 was produced in sufficient quantity in infected tissue there may have been insufficient antibody present to inhibit all activity. Antibodies raised against cell wall degrading enzymes have been successfully used in preventing disease. Anti-cutinase antibodies have been shown to prevent infection of pea stems by *Fusarium solani* f.sp. *pisi* and infection of papaya fruits by *Colletotrichum gloeosporioides* when mixed with the spores of these fungi (Kolattukudy, 1985). Cutinase had previously been demonstrated to be secreted by the spores of these fungi on the host surface. Alternatively the Xyl1 antibody may have failed to reduce disease because Xyl1 is not essential. The large number of xylanase isoforms produced by *S.nodorum* in wheat cell wall-grown cultures suggests that these may be able to compensate for loss of Xyl1 activity. To date no xylanases have been demonstrated to be essential for pathogenicity. Triple and double gene disruption mutants of xylanase genes in *C.carbonum* and *M.grisea* respectively (Apel-Birkhold and Walton, 1996; Wu *et al.*, 1997) both failed to reduce disease. In both cases additional xylanase isoforms were detected that had not been observed previously.

5.2.5 Wheat cell wall degradation by Xyl1

The biochemical characterisation of Xyl1 suggests that it may be a family 11 xylanase, a group of xylanases with a much higher substrate specificity than family 10. Family 11 xylanases are unable to cleave the xylan backbone next to substituted residues (Biely *et al.*, 1997). This suggests that this family may be more dependent on accessory enzymes to remove side groups before they can act. Hence it may be expected that these xylanases are less able to degrade the plant

cell wall arabinoxylan in isolation. Indeed, not all xylanases are able to degrade arabinoxylan alone and require the synergistic action of other accessory enzymes. For example, the xylanases II and III from *Talaromyces emersonii* were unable to hydrolyse arabinoxylan unless the polymer had been previously treated with an α -arabinofuranosidase of the same organism (Sunna and Antranikian, 1997).

However, Xyl1 was able to degrade the wheat cell wall by itself. Wall degradation was only measured for 5h and maximum hydrolysis of the wall by Xyl1 had not been reached. It is unlikely that extensive hydrolysis of the wall by Xyl1 would be possible, because the level of hydrolysis would probably be restricted by the number and distribution of xylose residues with substituents. In which case the maximum level of wall hydrolysis may be increased in the presence of accessory enzymes. Alternatively, it may be that this enzyme can act alone and may possess deacetylation (Laurie, *et al.*, 1997) or arabinosidase activity (Matte and Forsberg, 1992). However, this is unlikely because small xylanases, such as Xyl1, tend not to possess these extra domains (Black *et al.*, 1994). It is clear that Xyl1 can degrade the wheat cell wall on its own, releasing smaller oligosaccharides that may be acted on by other xylanases or accessory enzymes. The ability of xylanases to release reducing sugars from the wheat cell wall has also been reported for a number of other fungal xylanases. A xylanase from the plant pathogen *Verticillium albo-atrum* was able to release reducing sugars from isolated tomato stem walls (Cooper *et al.*, 1978). Also, a xylanase from *Trichoderma pseudokoningii* released carbohydrate containing primarily xylose and arabinose from corn cell walls (Baker *et al.*, 1977).

As well as reducing sugar release, a strong increase in U.V. absorption was also detected following the action of Xyl1 on the wall. This suggested that phenolic-linked polysaccharides were being released as a result of xylanase degradation. Comparison with standard absorption spectra and the blue fluorescence at 366nm indicated that this increase in absorbance may result from the release of ferulic-acid linked xylooligosaccharides. Ferulic acid is ester linked to the 0-5 of arabinosyl units in arabinoxylan and through its ability to form diferulic acid

cross links between neighbouring xylan molecules is thought to play an important role in cross-linking xylan molecules into the wall (Carpita and Gibeaut, 1993). Such cross linking is thought to stabilise the wall and make it more resistant to microbial wall degrading enzymes (Ikegawa, *et al.*, 1996). Degradation of these cross-links would therefore be advantageous to a pathogen in increasing the susceptibility of the wall to attack by other CWDE. Hence, micro-organisms are known to produce ferulic acid esterases which release ferulic acid from arabinoxylans (Jeffries, 1990). Only certain ferulic acid esterases can act alone on a preparation of the cell wall to release ferulic acid. Others require the additional activity of a xylanase and all ferulic acid esterase activities are increased in the presence of xylanases. Xylanases solubilise feruloylated oligosaccharides which are better substrates for ferulic acid esterases than the insoluble cell wall (Bartolome *et al.*, 1997). There have been few reports of the release of diferulic acid dimers from cell walls by ferulic acid esterases. These dimers are the cross-links between xylan molecules and are much more resistant to enzymic attack. However, recently a ferulic acid esterase from *A.niger* together with a xylanase have been shown to release a ferulic acid dimer from wheat and barley cell walls (Bartolome *et al.*, 1997). This ability was greatly enhanced by previous solubilisation by xylanase activity.

The fact that Xyl1 is an endo-acting xylanase capable of degrading the wall alone, without the synergistic action of accessory enzymes indicates that this is a potentially destructive CWDE. Accessory enzymes such as acetylesterase and arabinosidase, detected in the culture filtrate of *S.nodorum* (Chapter 3) may enhance the activity of Xyl1. The release of ferulic acid-linked xylooligosaccharides as a result of Xyl1 action will enhance the ability of ferulic acid esterases to disrupt wall structure by removal of ferulic acid groups and the degradation of dimer cross links, hence making the wall more susceptible to further degradation by other CWDE. Ferulic acid esterase was not assayed for in this work and hence could be present in the pure Xyl1 preparation used to test the ability of Xyl1 to degrade the wheat cell wall. Its presence *in vitro* and *in vivo* and its possible synergism with Xyl1 should be investigated.

5.2.6 Effect of Xyl1 on tritordeum suspension cells

Xyl1 failed to kill tritordeum suspension cells, despite the known ability of Xyl1 to degrade wheat cell walls. Pure SNP1 did not kill tritordeum suspension cells (Chapter 4) and no synergistic effect was observed when SNP1 was used in conjunction with Xyl1. Crude culture filtrates from cell wall grown cultures were also ineffective at killing the suspension cells. This crude filtrate contained a whole range of CWDE and presumably oligosaccharides derived from the wheat cell wall as a result of their action. As discussed in Chapter 4, tritordeum is a cross between wild barley and durum wheat (Barcelo *et al.*, 1993) and is more resistant to infection by *S.nodorum* than wheat (Rubiales *et al.*, 1996). Hence, there may be differences in cell wall structure that may prevent Xyl1 from degrading it. Ideally therefore these enzymes should be tested against wheat cell suspensions, or cells in wheat leaves. Alternatively, the concentrations of enzymes used in this study may not be as high as those produced locally during contact between fungal hyphae and the plant cell wall (Xu and Mendgen, 1997).

As discussed in Chapter 4, the nature of the suspension cells combined with the use of FDA as a viability stain only allowed extensive cell death to be detected. The suspension cells generally comprised small clumps of several cells and this may have made them more resistant to enzymic attack, but may also have prevented detection of the death of inner cells. It may have been possible to sieve cells through a size filter in order to obtain only single cells or groups of two or three cells. Any dead cells would then have been easier to identify. The use of the fluorescent viability stain FDA may be complicated by naturally occurring fluorescent compounds in the cells giving false positives (Baker and Mock, 1994). The level of staining by FDA is also difficult to assess, as some cells fluoresce more brightly than others and hence cell death is difficult to quantify. It may have been possible to obtain a more accurate measurement of cell death by also using the stain Evans blue (Baker and Mock, 1994).

It may be of course that the xylanase, Xyl1, is incapable of killing suspension cells, alone or in combination with the protease SNP1. An endo-xylanase from *T.viride* which could solubilise arabinoxylans in cell walls could not macerate graminaceous tissues (Ishii, 1988). In the same study this xylanase was also incapable of killing rice suspension cells, whereas another xylanase from *T.viride* could.

Some cell wall degrading enzymes have been found to be toxic to plant cells or induce defence responses (Walton, 1994). In many cases it is the oligosaccharides released by the action of the enzyme and not the enzyme itself that is recognised by the plant. The best studied of these are the oligalacturonides which can induce the production of phytoalexins, activated oxygen and proteinase inhibitors (Walton, 1994). Purified PL and PME from *M.grisea* cultures grown on rice cell walls could not kill maize suspension cells (Bucheli *et al.*, 1990). However, neither could fragments released by their action on isolated maize walls. Instead, an unidentified heat-labile factor in culture filtrates released heat-stable factors from maize walls that were capable of killing maize suspension cells. The heat-labile component of culture filtrate was composed of more than one protein; it is possible that the synergistic action of more than one CWDE is required to generate cell wall fragments that will elicit a resistance response. This may give the plant cells more control over the response if elicitation by two factors is required; this would limit erroneous elicitation and would localise the response to the site of both enzymes. Xylanase activity, in contrast to PL and PME, was found to release some heat-stable killing activity from isolated maize walls and is thought to be at least one of the enzymes responsible for this activity in culture filtrates. Xylanases have also been shown to be directly active against cells, not acting *via* the release of wall fragments. Xylanase activity was found to be responsible for the death of suspension-cultured rice cells during attempts to isolate protoplasts (Ishii, 1988). Treatment of suspension cells of tobacco with a xylanase from *T.reesei* and *T.viride* was found to induce hypersensitive-like cell death (Yano *et al.*, 1998). Elicitation did not result from release of wall fragments but was due to specific recognition of

these xylanases and not others, from *Bacillus cirulans* and *B.subtilis*. Thus xylanase-induced death was not a general toxic effect but was instead due to a specific cellular signal-transduction cascade that ultimately results in hypersensitive cell death. Xylanases from *T.viride* have been previously shown to induce defence responses in leaves of tobacco (Bailey *et al.*, 1990). It has also been proposed that the xylanase itself is the elicitor (Sharon *et al.*, 1993) and elicitation by the xylanase has been demonstrated to be cultivar specific, under the control of a single dominant gene (Bailey *et al.*, 1993). This suggests that in this system, detection of xylanase may play a role in specific disease resistance. Such elicitation of defence responses was not observed in barley cell suspension cultures following treatment with the β -1-,4-xylanase produced by *Bipolaris sorokiniana* (Peltonen *et al.*, 1997). Interestingly, this same xylanase was able to elicit defence responses in suspension cells of the non-host parsley. In contrast, a thermostable xylanase from *Clostridium thermocellum* has successfully been expressed at high levels in the apoplast of transgenic tobacco with no detrimental effect on the plant (Hebers *et al.*, 1995). However, the temperature optimum of the xylanase was 70°C and it only retained 20% of its activity at the 25°C growth temperature of the tobacco.

Detection of CWDE and their products by plant cells has therefore been demonstrated to be important in plant cell defence. This suggests that CWDE production by pathogens may be important in pathogenicity; avoidance of plant defence responses must be a prerequisite for pathogenesis by most plant parasitic microorganisms. Hence some pathogens may have evolved less efficient CWDE because destructive, extracellular endo-glycanases are more likely to elicit defence responses more rapidly. In a study by Cervone *et al.* (1987) an endo-polygalacturonase was purified from *Aspergillus niger* that was able to release cell wall pectic fragments and elicit a necrotic response in cowpea (*Vigna unguiculata*). The purified PG exhibited a specific activity considerably higher than those of PGs purified from phytopathogenic fungi that successfully attack and colonise living plants. Cervone *et al.* (1987) suggest that the fact that *A.niger* acts only in post-harvest decays and is not able to attack field plants may

be due to the action of this highly efficient PG inducing plant defence responses. In contrast, plant pathogens with less efficient PGs may be able to avoid or delay elicitation of host defence responses.

It was concluded from this work that *S.nodorum* is capable of producing a number of different isoforms of xylanase activity *in vitro* when grown on wheat cell walls. All but one of these xylanases appeared to have a low pI. The high pI xylanase, Xyl1 was expressed *in vivo* and is capable of degrading the wall. The abundance of different isoforms may make this system hard to resolve genetically and hence gene disruption for fungicide target validation remains a difficult goal.

Chapter Six: General Discussion

Successful colonisation of the host by plant pathogenic fungi may require degradation of the plant cell wall which can also serve as a nutrient source. The work described in this thesis clearly demonstrates the ability of *S.nodorum* to produce an array of plant cell wall degrading enzymes *in vitro* when grown on isolated wheat cell walls. The extensive range of cell wall degrading enzymes produced reflected the heterogeneity and composition of the wheat cell wall and the possible synergistic action between these enzymes required to degrade it. These included, xylanase, laminarinase, cellulase, protease, arabinosidase, acetylesterase, β -glucosidase and β -xylosidase. The low levels of polygalacturonase produced combined with the absence of any pectin degrading enzymes reflected the very low levels of these types of polymers in monocot cell walls (Carpita and Gibeau, 1993). In contrast, one of the most abundant enzymes, xylanase, reflected the typical predominance of xylan in wheat cell walls (Carpita, 1996). In addition to xylanase, another highly induced enzyme *in vitro* was protease and both of these enzymes were also detected at elevated levels in extracts from infected tissue. This work clearly indicated the value of using the host cell wall as an *in vitro* carbon source in allowing identification of fungal enzymes with potential importance *in planta*.

All of the cell wall degrading enzyme activities detected in liquid culture were also present in cirrus mucilage, produced *in vitro*. The presence of these enzymes in the absence of their inducing substrates suggested that the production of these enzymes in cirrus mucilage was developmentally regulated. This clearly contrasts with many other CWDE which show substrate induction and catabolite repression (Cooper, 1983). If this mucilage remains associated with spores following dispersal the 'pre-formed' cell wall degrading enzymes may also be present and available to the spore during early stages of infection to assist in spore adhesion or host penetration.

Further characterisation of the xylanase activity detected *in vitro* revealed that there are a large number of isoforms of xylanase contributing to this activity, consistent with a previous report of thirteen different forms (Lehtinen, 1993). The partial purification of a high pI endo-acting xylanase (Xyl1), not detected by this previous study, indicates that there may be more, as yet uncharacterised, xylanase isoforms. Antibodies raised to Xyl1 enabled this xylanase to be distinguished from other xylanase forms *in vitro* and to be detected *in planta* during the later stages of infection. Which, if any, of the other xylanase forms detected *in vitro* are also produced during host infection is not known. The use of the antibody to Xyl1 showed that it is not present in cirrus mucilage and hence other xylanase isoform (s) must be responsible for the activity detected. This suggests that different isoforms with different substrate specificities and/or regulation may be required at different stages of infection.

A role for xylanases in cell wall degradation and host colonisation by a wheat pathogen is likely given the abundance of xylan in the wheat cell wall. Xyl1 was clearly able to degrade the wall alone, without any prior action by other enzymes which can act on xylan, such as acetylsterase, arabinosidase or α -glucuronidase. This indicates that Xyl1 may be a very destructive enzyme during infection of wheat by *S.nodorum* and combined with its detection during infection this enzyme might be an important pathogenicity factor. However, its production in the later more necrotrophic stages of infection may suggest a more saprophytic role for this enzyme and to date, no xylanases have been successfully demonstrated to be essential for pathogenicity. Triple and double mutants of xylanase genes in *C.carbonum* and *M.grisea* respectively (Apel-Birkhold and Walton, 1996; Wu *et al.*, 1997) have only led to detection of new isoforms not observed previously. Only once all xylanase isoforms in these pathogens have been disrupted will their importance in disease be determined. The implication for xylanases in disease therefore remains purely circumstantial, as described in this thesis for Xyl1.

Characterisation of the protease activity detected *in vitro* revealed a single isoform of a high pI trypsin-like protease (SNP1) and two low pI forms of aspartic protease. Purification and characterisation of SNP1 revealed that this was a typical trypsin-like protease cleaving the carboxyl side of arginine and lysine residues. Cloning of the *SNP1* gene indicated that it is synthesised as a prepropeptide and contains the conserved trypsin active site region and catalytic triad. Characterisation of a trypsin-like protease found in infected wheat combined with western analysis using antibodies raised to SNP1 enabled this enzyme to be detected throughout infection and in cirrus mucilage. Inhibition of aspartic and trypsin-like protease activity with commercial inhibitors reduced disease suggesting that both proteases may play a role in infection by *S.nodorum*. The fact that inhibitors against these two different classes of proteases were both effective at reducing disease suggests that these two proteases with very different pH optima and substrate specificities are complementary and cannot replace each other. The effect on disease of inhibition of both protease classes with a mixture of inhibitors needs to be investigated. The evidence for the importance of SNP1 in disease is particularly strong because in addition to its detection in lesions, the most effective reduction in disease occurred with the trypsin inhibitor, aprotinin. In addition, infiltration of wheat leaves with the anti-SNP1 antibody also significantly reduced disease symptoms. Studies on the importance of proteases in the pathogenicity of plant pathogens have shown that at least some proteases appear essential for disease (Ball *et al.*, 1991; Dow *et al.*, 1990). In other cases where pathogenicity was unaffected, residual protease activity remained (Robertson, 1984; Murphy and Walton, 1996). The reasons for these discrepancies may be that proteases, in contrast to xylanases, could play a different role in each host-pathogen system studied and in some cases these may not be essential for disease. SNP1 has been studied here as a potential cell wall degrading enzyme, and indeed has been shown to be capable of acting as such, without synergistic assistance from polysaccharide-degrading enzymes. It is possible however, that SNP1 plays other roles *in planta* such as enzyme, toxin or elicitor processing, in countering host defence responses or in nutrition instead of, or in addition to, wall degradation. The role for the aspartic protease also

produced by *S.nodorum* is unknown, although its optimal induction by wheat cell walls indicates that this may be another potential wall degrading protease.

The central aim of this thesis was to determine whether any one plant cell wall degrading enzyme might be essential for pathogenicity and hence be a suitable fungicide target. The circumstantial evidence presented in this thesis suggests that Xyl1 and SNP1 play important roles in disease, and that SNP1 may be essential. Cloning of the *SNP1* gene now makes testing this theory by gene disruption possible. Circumstantial evidence continues to be an important part of our understanding of the importance of cell wall degrading enzymes in disease, particularly while studies using gene disruption continue to be inconclusive in many cases (Oliver and Osbourn, 1995). The presence of residual enzyme activity or the induction of new isoforms following gene disruption complicates assessment of the role of a single enzyme in pathogenicity. Even where residual activity is not detected *in vitro* it may be that the assay is too insensitive to detect all residual activity or that there may be more forms of the enzyme that are specifically induced *in planta*. Alternatively, there may be long term effects on pathogenicity that are not observed in the pathogenicity test employed. It is also possible that a single enzyme will not be essential for disease. If a cocktail of CWDE is required instead then disruption of a number of wall degrading enzymes may be required to see any effect. In this context, fungicides directed against a single CWDE are unlikely to give adequate, durable control. In particular, the use of protease inhibitors in a field situation seems unfeasible because of the ubiquitous presence of proteases in other organisms; their use would only be possible if inhibitors specific to fungal proteases were found. Instead, fungicides directed at preventing secretion or induction of a number of CWDE might be more effective (Wattad *et al.*, 1995; Milling and Richardson, 1995; Kotoujansky, 1987). An alternative to fungicide development is the production of transgenic plants expressing single chain antibodies to one or a number of wall degrading enzymes, or expressing fungal-specific protease inhibitors. Finally however, if the functional redundancy theory of Van Etten *et*

al. (1994) is correct, only once all methods of penetration are inhibited, i.e. mechanical and enzymic will any effect be observed.

6.1. Future work.

Given the circumstantial evidence implicating SNP1 in disease the most obvious continuation of this work is disruption of the *SNP1* gene to produce a null mutant lacking in SNP1 activity. SNP1 apparently exists as a single isoform and only one trypsin-like protease encoding gene has been detected, hence disruption of the *SNP1* gene may result in the loss of all trypsin-like protease activity. If no residual or additional activity is detected this will allow the importance of SNP1 in disease to be determined.

Generation of internal sequence from the N-terminally blocked Xyl1 protein will allow the gene encoding this enzyme to be cloned and disrupted also. However, this is unlikely to be successful at reducing disease, given the large number of other xylanase forms detected *in vitro* that may be able to compensate for the loss of Xyl1. The complexity of the xylanases produced by *S.nodorum* may be difficult to resolve, but targeting those isoforms which are also produced *in vivo* may be an initial step. Identification of these forms could be achieved by using IEF activity gel overlays on extracts from infected plants.

Further work is also required to determine whether the aspartic protease is produced during infection of the plant and what its role in disease might be. For example can it also degrade the plant cell wall? Is it toxic to host cells? The effective reduction in disease by pepstatin implicates a role for aspartic proteases in disease. Purification and characterisation of the two aspartic isoforms combined with the complete cloning of the aspartic protease gene identified by AgrEvo Uk will enable the number of genes encoding these proteases to be determined and their gene (s) to be disrupted.

The creation of triple mutants in both proteases and Xyl1 may result in a more effective reduction in disease, or merely induce or reveal yet more uncharacterised isoforms. The use of antisense to inhibit these enzymes may provide a more realistic view of the potential to control *S.nodorum* infection by inhibition of these enzymes, because many fungicides will not achieve complete inhibition of their target (Caten and Holloman, 1995).

Detailed studies with any gene disruption mutants obtained will be required to determine the effects on pathogenicity, as many studies have shown that subtle differences in pathogenicity may go undetected if the wrong inoculation conditions are used (Dow *et al.*, 1990; Rogers *et al.*, 1994). For example, it would be relevant for *S.nodorum* to use low inoculum levels to simulate spore numbers likely to be encountered in the field situation (Griffiths and Peverett, 1980). Also, although detached leaf pathogenicity tests are convenient and sensitive, ultimately putative mutants must be tested on vigorous, intact host plants. Improved tests are also required to compare fitness of the mutants with the wild types such as ability to produce spores. Even if lesion size is the same in the primary inoculations the number of spores produced in that lesion may be more critical to future disease cycles. Mutants should also be compared to wild type for fitness throughout the disease cycle, including adhesion and dispersal of spores and their ability to survive desiccation, starvation and winter.

Further studies using commercial inhibitors may also provide more information on the role of the proteases in disease. Aprotinin and pepstatin could be used in combination to study the effect of inhibition of both proteases. More detailed studies to investigate the effect of inhibition of these proteases could look at their effect on the biomass of *S.nodorum* produced during infection and transmission and scanning electron microscopy could be used to investigate the effects on fungal morphology in more detail than the light microscopy used in this thesis.

The anti-SNP1 antibody can now be used to track whether this and possibly other CWDE present in cirrus mucilage remain associated with spores following dilution and dispersal. It may also be possible with this antibody to detect more specifically the timing of production of SNP1 during early stages of infection such as germination and appressoria formation. Immunolocalisation of both SNP1 and Xyl1 using gold-labelled antibody may allow the exact site of production of these enzymes *in planta* to be detected. Alternatively, the fusion of the *SNP1* promoter to the green fluorescent protein would allow the timing of production of SNP1 *in planta* to be observed directly. Northern analysis could also be used to investigate the timing of *SNP1* expression *in planta*.

Further analysis of the *SNP1* promoter may allow regions essential for carbon and nitrogen regulation to be identified. CREA or AREA proteins from *A.nidulans* could be used in band shift assays to see if they bind the *SNP1* promoter *in vitro*. If homologous regulatory pathways exist in *S.nodorum* these regulators could be cloned and disrupted and the effect on *SNP1* regulation determined. Point mutations in putative CREA and AREA binding sites may also identify whether these are essential for carbon and nitrogen regulation of *SNP1*. If not, then there may be alternative regulatory pathways, perhaps specific for pathogenicity genes, as is suggested for *M.grisea* (Lau and Hamer, 1996). A more detailed analysis of the regulation of SNP1 activity *in vitro* using other protein sources is also required to see if it is specifically induced by hydroxyproline-containing proteins. This could be done by assay or Northern blot analysis. The possible involvement of cAMP in induction of the *SNP1* gene requires further investigation and the importance of the putative cAMP responsive elements found in the *SNP1* promoter could be tested by point mutation.

The ability of SNP1 to degrade the different types of proteins in the wheat cell wall should be tested to determine the specific site of action of SNP1, and combined with ultrastructural localisation of the enzyme, this might determine whether this is its true physiological role. Analysis of the peptides solubilised

from wheat cell walls may also indicate which proteins are acted on by SNP1 and what linkages are cleaved by this enzyme. The ability of SNP1 to degrade isolated cell wall proteins from other cereals could also be tested and whether SNP1 can degrade the more heavily substituted wall proteins of dicotyledons.

The ability of SNP1 to degrade host and non-host walls would also be of interest in determining whether wall degradation by SNP1 could be correlated with host range. The ability of wheat, barley and grass isolates of *S.nodorum* to produce an SNP1-like protease *in vitro* and *in planta* requires investigation, and if so can these SNP1-like enzymes degrade host and non-host wall proteins. Also, the levels of production and activity of these SNP1-like proteases should be studied to see if they correlate with virulence in these isolates.

The normal production of active forms of all cell wall degrading enzymes in any *SNP1* disrupted mutants should be confirmed in case its role is in zymogen activation. Other possible roles for SNP1 in pathogenesis require investigation, such as whether it is toxic to wheat protoplasts, alone or in conjunction with wall polysaccharidases such as xylanases or arabinosidases.

The possible synergistic action of SNP1 and Xyl1 could be investigated by determining whether degradation of the wall by SNP1, detected early during infection, enhances the degradation of the wall by Xyl1, detected later during infection. Such synergy was observed between an aspartic protease and an endopectin lyase in *Botrytis cinerea* during infection of carrot (Movahedi and Heale, 1990b).

Finally, further characterisation of the other cell wall degrading enzymes produced by *S.nodorum* may identify more enzymes which, even if they are only produced at low levels, such as arabinosidase, may play essential synergistic roles in pathogenicity. For example, action by this enzyme may be essential for some xylanases and proteases to act because substrates for both enzymes in intact walls possess arabinose side chains. In addition, new studies to look for other wall

degrading enzymes relevant to wheat cell wall structure, such as ferulic acid esterase may find new enzymes that could be important in the pathogenicity of plant pathogens. The search for new and different CWDE must continue to expand as studies of plant cell wall structure continue to find new types of cell wall polymers and linkages.

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